Cross acclimation

the effect of prior acute and repeated heat exposures on physiological responses and performance in acute normobaric hypoxia

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ABSTRACT

Abstract

The independent effects of acute heat and hypoxic stress on human physiological function and performance are relatively well documented. Although in the field these environmental stressors rarely occur in isolation the effects of combined or sequential exposure to them has not been extensively studied in humans. Animal models have however shown that acclimation to one stressor can induce 'cross acclimation' a positive adaptive response upon exposure to a different stressor. The three studies within this thesis were conducted in humans to assess how exposure to acute and repeated exposures to heat affects the later physiological and cellular responses to acute exercise in normobaric hypoxia. A possible site for any cross-acclimatory affects and conferred cellular tolerance resides in the heat shock response (HSR) and the increased expression of heat shock proteins (HSPs). The 72 kilodalton HSP, HSP72 has been implicated in heat acclimation mediated cross acclimation in rodent models, and also shown to be important in the human adaptation to heat and hypoxic stressors.

Study One determined the physiological and HSR to exercise in both heat (HEAT; 40°C) and hypoxia (HYP; F_IO_2 0.14) alone, and in combination (COM) as well as a normothermic normoxic control (NORM). 24 hours after the initial exposure a hypoxic stress test (HST; 15 minutes of seated rest and 60 minutes of cycling exercise at 50% normoxic $\dot{V}O_{2\ peak}$) was conducted to determine what effect the prior stress exposure had on both whole body physiological responses and the cellular HSR. It was hypothesised that the stressor that elicited the greatest physiological strain and HSR on day one would have the biggest effect on reducing physiological strain in a subsequent HST. Twelve male participants completed 4 trials consisting of a 15 minute rest period in normoxic temperate conditions, followed by 30 minutes seated rest and 90 minutes cycling exercise at 50% N$\dot{V}O_{2\ peak}$ within NORM, HEAT, HYP and COM. 24 hours after completing this exercise bout, participants undertook a HST. Exercise duration was reduced in HEAT (78 ± 12mins), HYP (81 ± 13mins) and the CON (73 ± 19mins) trial compared to the NORM (89 ± 3mins). HR and core body temperature (T_{core}), and thus physiological strain, were greater in the HEAT and COM trial compared to HYP alone. This response was also observed with post exercise monocyte HSP72 (mHSP72). Basal HSP72 was elevated 24 hours after the HEAT and COM and attenuated post HST. Exercising HR, T_{core} and PSI was reduced during the HST 24 hours after a heat stressor had been applied, but unaffected by a prior hypoxic exposure. Therefore the hypothesis was accepted. It was concluded that at the temperature and level of hypoxia studied, a prior exposure to exercise heat stress was beneficial when conducting subsequent acute hypoxic exercise.

Study Two investigated the effect of short-term heat acclimation (STHA) on subsequent hypoxic tolerance in 16 male participants divided equally into 2 matched groups. This study also examined the response of extracellular HSP72 (eHSP72) to acute hypoxic exercise. It was hypothesized that STHA would increase basal HSP72

and that the post HST increase in HSP72 would be attenuated in this group, indicating conferred cellular tolerance. Eight males completed a HST one week before undertaking 3 consecutive days of STHA (60 min/day, 40°C, 50% $\dot{V}O_{2\ peak}$) followed by a final HST 48-hours after the last acclimation day. The matched controls (CON) completed an identical protocol in normothermic, normoxic conditions. The initial HST induced a post exercise increase in HSP72 in both groups. HSP72 was increased after the first day of heat acclimation and unchanged in the control group. After acclimation day 2, basal HSP72 was increased from on day 1 basal values and the post exercise increase observed on day 1 was absent in the heat group. The increase in basal HSP72 persisted until the post acclimation HST for the STHA group and post exercise HSP72 was attenuated. eHSP72 increased immediately after the HST in both groups, however large inter-individual variation was evident. Mean exercising HR, T_{core} and physiological strain was reduced during the HST in the STHA group, indicating that a short period of heat acclimation can improve both cellular and physiological tolerance to exercise in acute normobaric hypoxia.

Study Three examined how a prior period of long term heat acclimation (LTHA) or time and absolute exercise intensity matched hypoxic acclimation (HA) affects both tolerance and performance to a HST and 16.1 km time trial (TT). Plasma hypoxia inducible 1 alpha (HIF-1α) was assessed before and after the acclimation periods as this transcription factor plays an important role in heat acclimation mediated cross tolerance. Twenty-one male participants completed ten 60-minute cycling bouts (50% N $\dot{V}O_{2\ peak}$) in thermoneutral, normoxic conditions (CON, 18°C, F_IO_2 0.209**;** n = 7), heated conditions (LTHA, 40°C, n = 7) or hypoxic conditions (HA, F_IO_2 0.14, n = 7). A HST immediately followed by a 16.1 km TT was completed one week before and 48 hours after the acclimation period. Both LTHA and HA induced increases in basal HSP72 by the end of the 10-day period. Increases in basal HSP72 occurred earlier in the acclimation period and to a greater magnitude with LTHA. Prior to the post acclimation HST both basal HSP72 and plasma HIF1-α were elevated in the LTHA and HA groups, with no changes observed in CON compared to the initial HST. Post HST mHSP72 and HIF1-α was attenuated in LTHA and HA. Mean exercising HR, T_{core} and PSI were reduced in the LTHA group with no changes in these physiological variables observed in the HA or CON groups. During the TT, mean power output (MPO) was elevated at each kilometer in the HA group, leading to an improved performance after acclimation. The LTHA group produced greater power outputs between km 1 – 8 and 14-16 and consequently were faster overall compared to their pre acclimation TT. This indicates an altered pacing strategy following the LTHA period. The data suggests that, at the levels studied herein, LTHA induces a faster accumulation of basal mHSP72 over a 10-day period, occurring to a greater magnitude. This is the first study to examine the plasma HIF-1α response to both heat and hypoxic acclimation in humans. The data suggest that each environmental stressor induces an increase in resting levels of this transcription factor, however further study is required due to the large variation in response. It is not yet known

whether the benefits conferred from heat to acute bouts of hypoxia would translate to more prolonged hypoxic exposures. Both the mechanisms of cross-acclimation and the effects of extended or prolonged hypoxic exposure following heat acclimation require further study.

The immediate post exercise mHSP72 increase to exercise was consistently shown to be greater following a heat stress condition when compared to hypoxia. STHA induced greater increases in basal mHSP72 compared to the acute exposure, further attenuating post HST mHSP72 elevations and physiological strain. LTHA increased basal mHSP72 at a faster rate and magnitude than HA and 16.1km time trial performance improved to a similar magnitude following both heat and hypoxic acclimation It is speculated that heat acclimation mediated activation of HIF-1α may hold a key mechanistic role in the observed cross-acclimatory response. From a practical perspective, the use of heat-stress based acclimation/training programs may provide a cheaper and more effective means of preparing individuals for subsequent hypoxic exposure. Future studies should confirm these observations hold true in a hypobaric environment and establish how prior heat acclimation may impact on longer term exposures and adaptations to hypoxic environments.

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List of abbreviations

ADP	Adenosine diphosphate
ADR	Adregenic receptor
AMS	Acute mountain sickness
ANOVA	Analysis of variance
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
a-VO_2	Arterio-venous oxygen
BTPS	Body temperature pressure saturated
CBP	CREB-binding protein
C_aO_2	Arterial oxygen content
CO_2	Carbon dioxide
CV	Coefficient of variation
Δ PV	Change in plasma volume
D_{BP}	Diastolic blood pressure
D_{MAX}	Maximum distance from the line of best fit
2-3 DPG	2,3-diphosphoglycerate
DMOG	Dimethyloxalyglycine
EDTA	Ethylenediaminetetraacetic acid
eHSP72	Extracellular heat shock protein 72
EO	Effector organ
EPO	Erythropoietin
EPO_r	Erythropoietin receptor
FADH	Flavin adenine dinucleotide
FIH	Factor inhibiting hypoxia inducible factor
F_ECO_2	Fraction of expired carbon dioxide
F_EO_2	Fraction of expired oxygen
F_IO_2	Fraction of inspired oxygen
GCPR	G-coupled protein receptors
GLUT	Glucose transporter
GRP	Glucose regulating protein
H^+	Hydrogen ion
Hb	Hemoglobin
HCT	Hematocrit
HCO_3^-	Bicarbonate
HIF	Hypoxia inducible factor
HR	Heart rate
HRE	Hypoxic response element
HSC	Heat shock cognate
HSE	Heat shock element
HSF	Heat shock transcription factor
HSP	Heat shock protein
HSR	Heat shock response

LIST OF ABBREVIATIONS

HST	Hypoxic stress test
HST-TT	Hypoxic stress test and 16km time trial
HVR	Hypoxic ventilatory response
IGF-1	Insulin-like growth factor 1
IHE	Intermittent hypoxic exercise
IL-6	Interleukin 6
IL-10	Interleukin 10
kDa	Kilodalton
LHTL	Live high train low
LTHA	Long term heat acclimation
mmHg	Millimeters of mercury
MAP	Mean arterial pressure
MCT	Monocarboxylate transporter
mHSP	Monocyte heat shock protein
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NOS	Nitric oxide synthase
O_2	Oxygen
PaO_2	Partial pressure of oxygen in arterial blood
$PaCO_2$	Partial pressure of carbon dioxide in arterial blood
PBMC	Peripheral blood mononuclear cell
PCr	Phosphocreatine
PFK	Phosphofructokinase
pH	The acidity or alkalinity of a solution on a logarithmic scale
PHD	Prolyl-hydroxylase
PO_2	Partial pressure of oxygen
P300	Transcriptional co-activating protein
Q	Cardiac Output
RBC	Red blood cell
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
RT-PCR	Real time polymerase chain reaction
SaO_2	Arterial oxygen saturation
SD	Standard deviation
SpO_2	Arterial blood oxygen saturation measured via pulse oximetry
SRC-1	Steroid receptor coactivator-1
STHA	Short term heat acclimation
STPD	Standard temperature pressure dry
SV	Stroke volume
TAD	Thiazole-4-carboxamide adenine dinucleotide
TAN	Total adenine nucleotides

LIST OF ABBREVIATIONS

TCA	Tricarboxylic acid
TGF	Transforming growth factor
T_{body}	Mean body temperature
T_{core}	Mean core temperature
T_{mu}	Muscle temperature
T_{skin}	Mean skin temperature
TNF	Tumour necrosis factor
TT	Time trial
UPS	Ubiquitin-proteome system
VCO_2	The volume of carbon dioxide expelled each minute
V_E	The volume of gas exhaled over one minute
VEGF	Vascular endothelial growth factor
VHL	Von-Hippel Lindau protein
VO_2	The volume of oxygen used within one minute
WBGT	Wet bulb globe temperature

1.0 Introduction

Athletes, military personal, fire fighters, and mountaineers are often required to perform vigorous physical activity in adverse environmental conditions. More recently, staged sporting events have required athletes to compete in extreme environmental conditions where they are neither native nor resident. The rise in popularity of Ultra-endurance events such as the Marathon des Sables, Badwater 100, Race Across America and Mont Blanc Ultra-Trail, as well as the ongoing conflicts in Afghanistan and Iraq mean that more people are facing exposure to, and required to perform in, extremes of heat and altitude.

UK military serving in the aforementioned conflicts are subjected to summer temperatures in excess of 40°C. Additionally, personnel are required to wear protective clothing, including helmets, heavy clothing, body armor, and carry additional loads. This both increases metabolic heat production and reduces the effectiveness of cooling via evaporative heat loss. These factors are clearly implicated in the 849 cases of heat related injury in UK forces between 2002 and 2003 (Bricknell, 2003). Of these 766 were admissions to hospital, with 161 patients evacuated to the UK (Bricknell, 2003). Similar incidences were recently reported for the US forces with 508 casualties experiencing edema, syncope, exhaustion, and heat stroke in Baghdad, Iraq, Kandahar and Afghanistan, between 2006-2007 (Stultz et al., 2008). Military personnel may be required to engage in activities and sojourns to altitude at short notice, without sufficient time to acclimatize to the undertaking of arduous physical work in such conditions. This lack of preparation and time to adapt to an altitude can increase the incidence of acute mountain sickness (AMS), and result in a reduction in operational ability (Chen et al., 2008). Indeed, the incidence and severity of AMS in un-acclimatized military

personnel has been shown to increase from 20% to 70% when ascending to altitudes between 2000m and 3960m (Gallagher and Hackett, 2004).

Repeated exposures to a stress or stressors invoke adaptive mechanisms, which reduce the internal strain resulting from a similar repeat exposure (Adolph, 1956). Both athletes and military personnel utilize heat and altitude acclimation protocols to augment their performance during high ambient temperatures or altitude (Muza et al., 2007). Altitude/hypoxic training, particularly the "live high train low" model (Levine and Stray-Gunderson, 1997) has been used frequently to purportedly improve exercise performance at sea-level, and recently repeated bouts of exercise heat stress have also been shown to improve exercise economy and performance in temperate conditions (Lorenzo, et al., 2010). This illustrates that adaptation to extreme environments can confer advantages in other situations, such as sporting performance. Adaptation, or acclimation, to a single stressor invokes a general pattern of responses, which have the potential to augment or interfere with acclimation to a second stressor (Fregly, 1996). This process has been termed *cross-acclimation*, defined as the influence of the earlier adaptation to one stressor on subsequent adaptation to a new environment (Piantadosi, 2007). Similarly, an acute one-off exposure to a stressor leads to a period, usually 1 – 2 days, during which there is protection from an otherwise lethal insult. This shorter-term protective exposure has been termed *preconditioning* (Sharp, 2004). Preconditioning responses are also possible between stressors if the stressors share some of the same generalized responses and adaptive mechanisms. (Horowitz, 2007).

One such shared generalized response common among exposure to different stressors is the heat shock response (HSR) and activation of a ubiquitous family of proteins, termed heat

shock proteins (HSPs). These proteins were discovered in *Drophosila Melanogaster* cultured in high temperatures (Ritossa et al., 1962). Members of this family are classed according to their size, ranging from 10 kiloDaltons (kDa) to 170 kDa. The highly stress inducible HSP72 has received considerable interest within the literature due to its involvement with conveyed cellular tolerance (Kalmer and Greensmith, 2009) and prominent role in preconditioning and cross-acclimation (Sharp et al., 2004; Maloyan et al., 2005; Horowitz et al,. 2007). A multitude of animal studies have established a role for HSP72 in inducing cellular tolerance to ischemic/oxidative stressors (Horowitz et al., 2005) and playing an important role in heat acclimation (Maloyan et al., 1999). In humans the evocation of the HSR has recently been a target for therapeutic treatments *in vivo* in humans (Yogaratnam et al., 2007) and improving both hyperthermic and hypoxic exercise tolerance (Sandstrom et al,. 2008). The elevation of basal HSP72 via heat acclimation (McClung et al., 2008; Kuennen et al., 2010; Magalhaes et al., 2010; Hom et al., 2010) mechanical stress (Ascensao et al,. 2008), acute resting hypoxia (Taylor et al., 2010) and repeated daily resting hypoxia (Taylor et al., 2012) has been established *in-vivo* in humans. This shared cellular response between stressors offers the potential for pre-conditioning and cross-acclimation between heat and hypoxia in humans.

Experiments within this thesis exposed sea level dwellers acutely to dry ambient heat (40°C, 15-20% relative humidity) and/or moderate altitude the equivalent to ~3000m above sea level (ASL; F_IO_2 0.14) at both rest and during exercise. Each investigation incorporated a range of whole body and cellular physiological measurements. Aspects of both the acute and acclimatory responses to both heat and hypoxia including cardiorespiratory, metabolic and stress hormone responses that occur in response to these environments are outlined in Chapter 2.

CHAPTER 1. INTRODUCTION

Currently a gap in understanding exists regarding the physiological and cellular responses to combined and sequential exposures to different environmental stressors such as heat and hypoxia. To date no research has determined the cellular and whole body stress response to both acute heat, and acute hypoxia within the same cohort of participants. Doing so could further elucidate the nature of both the stress specific response, as well as the general component of such responses. Whether this initial exposure invokes a short term preconditioning response, and altered HSP kinetics, to a second stressor has also yet to be determined. Lastly, the effects of both short term and long-term adaptive periods to heat and/or hypoxic stress on whole body human performance in acute hypoxia are yet to be described by the literature.

The aims of this thesis are:

Experiment 1, Chapter 4.

- Investigate the physiological and HSP72 response to individual and combined acute heat and hypoxia stress at rest and during exercise within the same cohort of participants.
- Determine the magnitude of any preconditioning effect of individual and combined acute heat and hypoxic exercise stress on subsequent exposure to exercise in hypoxia at the whole body and cellular level.

Experiment 2, Chapter 5.

- Investigate the effect of the initial short term phase of heat acclimation on basal HSP72 concentrations.
- Investigate the effect of the initial short term phase of heat acclimation on circulating HSP72 concentrations

- Explore whether any such heat acclimation mediated changes in HSP72 may affect physiological and cellular tolerance to exercise in acute hypoxia.

Experiment 3, Chapter 6.

- Investigate the effect of once daily heat or hypoxia for ten consecutive days on basal HSP72 expression.
- Explore whether any such daily heat or hypoxia-mediated changes may affect physiological and cellular tolerance to a subsequent exposure to exercise in acute hypoxia.
- Examine the plasma HIF1-α response to once daily heat or hypoxia for 10 consecutive days.
- Examine whether once daily heat or hypoxia for ten consecutive days affects cycling time trial performance in acute hypoxic conditions

2.0 Literature review

2. 1 Overview of the physiological responses to high ambient temperatures and hypoxic environments

The stressors of heat and hypoxia both lead to the rapid disruption of homeostasis and activation of specific effectors aimed at ameliorating the level of disruption caused by these conditions. Heat and hypoxia share some common physiological responses upon immediate exposure. For example, cardiac output is elevated however the cause of this response varies. Exposure to hypoxia reduces the partial pressure of oxygen in the blood, leading to an increase in resting cardiac output to ensure adequate O_2 delivery to tissues (Naeije et al., 1982). Whereas resting exposure to heat can increase the requirement for skin blood flow (SkBF) from 5-10% of cardiac output, to 50-70% of cardiac output, depending on the severity of the heating model employed (Johnson and Proppe, 1996). This increased demand for SkBF is met by an increase in resting cardiac output, and the redistribution of blood from other tissues (Johnson, 2010). In both these environmental challenges, an elevated HR almost entirely mediates the increased cardiac output (Rowell, 1969; Naeije et al., 1982). This increase in cardiac output is partially instigated by parasympathetic nervous system (PNS) withdrawal, alongside increases in sympathetic nervous system (SNS) activity, albeit to differing degrees within heat and hypoxia. The result is a reduction in cardiac filling, thereby reducing stroke volume in both heat and hypoxic stress. Similarly, for a given level of exercise, HR is greater during exposure to altitude and high temperatures. The following sections will describe the cardiorespiratory and metabolic responses to heat and hypoxia. The commonalities will then be discussed (Section 2.2.5).

2.1.2 Cardiorespiratory responses to acute heat stress at rest

Among the various physical environmental stressors, temperature has been described as ecologically the most important, as it is the factor that is all pervasive in most environments and lacks spatial or temporal consistency (Cousins and Bowler, 1987). Humans, as homeotherms, can tolerate a range of core temperatures 3-4°C around a basal temperature of approximately 37°C (Benzinger, 1969). Human thermoregulation is a dynamic, distributed, multi-sensor adaptive system well equipped for the dissipation of heat, generated either in the body by exercise or absorbed from the environment in hot conditions (Werner, 2010). As environmental temperature and humidity increase, the body is challenged to maintain its core temperature. The majority of studies investigating the passive responses to heat stress typically heat the volunteer using a water perfused suit (Rowell et al., 1969, 1986; Brothers et al., 2009), by whole body immersion of the entire body or limbs in warm water, or by exposure of the individual to warm environmental conditions using a climatic chamber (Taylor, 2000; Taylor, 2006; Crandall and Gonzalez-Alonso, 2010).

Increased skin temperature (T_{skin}) is the primary mode by which internal temperature is elevated at rest. However the different methods result in very different magnitudes of increase in core temperatures (T_{core}). Increases range from 0.5°C to 2°C depending on the level of the initial stressor (Johnson and Proppe, 1996). An elevated T_{core} during passive heating has been shown to directly increase pulmonary ventilation (White, 2006). The magnitude of the increase in T_{skin} is more pronounced for a rising T_{core} than for an elevated, but stable T_{core} (White et al., 2006; Tsuji et al., 2012). Elevations in T_{core} by 1 - 2°C give a typical sensitivity of pulmonary ventilation from 2.0 – 4.0 $L \cdot min^{-1} °C^{-1}$. It has been proposed that the increase in ventilation is a thermoregulatory response aimed at selectively cooling the brain (White 2006), however temperature mediated hyperventilation has been shown to lead

to hypocapnia and a concomitant reduction in cerebral blood flow (Brothers et al., 2009), which would increase brain temperature due to the reduction in heat exchange (Nybo et al., 2002). The physiological significance of hyperthermic hyperventilation remains unclear (Tsuji et al., 2012).

The typical responses to passive heating include cutaneous vasodilation and the increase of skin blood flow from 300mL•min^{-1} under normal resting conditions towards 7500mL•min^{-1} (Rowell 1969, 1986). The stimulus for cutaneous vasodilation is derived from several locations within the neural thermoregulatory hierarchy, and therefore can be initiated by both reflex (T_{core}) and local (skin) temperature changes. Each leads to an increase in skin blood flow (Charkoudian, 2003). Passive heat stress has been shown to reduce central venous pressure (Rowell et al., 1969; Johnson and Proppe, 1996; Minson et al., 1998; Crandall et al., 1999; Peters et al., 2000; Keller et al., 2009). The reduction in central venous pressure (CVP) is likely due to the redistribution of blood from central circulation to the cutaneous vascular beds, a theory examined by Crandall et al., (2008). As well as a reduction in central venous pressure, the authors also reported reductions in thoracic (14 ± 2%), cardiac (18 ± 2%), hepatic (23 ± 2%) and splenic (27 ± 2%) blood volumes. This is in support of previous work that has described vasoconstriction in splanchnic and renal circulations, thereby reducing blood flow in these regions (Rowell, 1969; Minson et al., 1998). It has been suggested that the reduced ventricular filling pressures and reduced central blood volume alongside a preserved or slightly raised SV during heat stress increases the inotropic state of the heart (Johnson and Proppe, 1996; Cubin, 1999). Brothers et al., (2009) observed that passive heat stress led to unchanged left ventricular filling velocity, an unchanged early diastolic mitral annular velocity, and an unchanged ratio of blood velocity/mitral annular velocity during the early diastolic phase. These findings suggest that passive heating has a minimal effect on

diastolic function. However, when these findings are considered alongside the reported heat induced decreases in ventricular filling pressure and central blood volumes, they may indicate that diastolic function is actually enhanced during heat stress (Crandall and Gonzalez-Alonso, 2010).

Cardiac output increases alongside decreases in the vascular conductance of non-cutaneous beds in order to prevent any decreases in arterial blood pressure as a result of the pronounced increases in total vascular conductance associated with vasodilation (Crandall and Gonzalez-Alonso 2010). These responses result in either minimal increases or no change in arterial blood pressure (Rowell et al., 1969; Rowell et al., 1986; Minson et al., 1999). Resting cardiac output ($\dot{Q}$) has been shown to be as high as 13L•min^{-1} during a pronounced passive heating challenge. Approximately 50% of this value was estimated as being directed towards the skin (Rowell et al., 1986).

In summary, passive heat stress can lead to an increase in pulmonary ventilation provided the heat load is sufficient enough to elicit at least a 1°C increase in T_{core}. Falls are seen in central venous pressure and blood volumes of central sites as a result of thermoregulatory redistributions of blood to the periphery. Increases in skin blood flow and $\dot{Q}$ are common, with 50% of the increase in $\dot{Q}$ directed towards the skin, whilst vascular conductance of non-cutaneous beds is reduced. The elevations in $\dot{Q}$ are mediated by increases in HR while SV is only marginally affected. Lastly, diastolic function could plausibly be enhanced during passive heating as many indicators for this variable remain unchanged despite the decreases in ventricular filling pressure and central blood volume.

2.1.3 Cardiorespiratory responses to acute hypoxia at rest

Humans have evolved a sophisticated physiological network to maintain oxygen homeostasis at the tissue level that involves the capture, binding, transport and delivery of molecular oxygen (Giaccia et al., 2004). A critical feature of this network is the ability to sense and respond to low blood oxygen content. *In-vivo,* hypoxia reduces the partial pressure of oxygen within the arterial blood, thereby disturbing whole body homeostasis (Taylor and Pouyssegur, 2007). The level of disturbance depends on the degree and extent of hypoxia incurred. Ascents to altitudes of ~3000m above sea level (asl) reduces the ambient PO_2 from 159mmHg at sea level to 110mmHg. This results in resting arterial oxygen saturation (SaO_2) falling from 96% at sea level to approximately 90-92%.

During a hypoxic exposure, several chemosensing systems act together to rapidly modulate pulmonary ventilation and perfusion, as well as blood circulation to optimise the supply of oxygen to metabolising tissues. The ventilatory response is mediated by the peripheral chemoreflex, a reflex arc from the carotid bodies to the respiratory muscle effectors (Torrance, 1996). The hypoxic ventilatory response (HVR) is time dependant (Easton, 1996; Powell, 2007). Thus, if hypoxia is administered, the HVR can be divided into a two-phase response. An initial, rapid phase of immediate increased ventilation occurring over the first 0-5 minutes of exposure, followed by a second phase of slow decline (5-20 minutes; Steinback and Poulinm 2007), termed hypoxic ventilatory decline (HVD; Liang, 1997).

Unlike passive heating, which requires a threshold increase in T_{core} to be obtained before any ventilatory response is noted, the response to a reduced SaO_2 is rapid. However, HVR may not be seen until the partial pressure of oxygen in arterial blood (P_aO_2) is reduced to 50 or 60 mmHg, corresponding to altitudes over 4000m (West, 1982). The increase in ventilation

progresses to a hypocapnic state (Favret and Richalet, 2007), elevating blood pH, producing respiratory alkalosis. Such alkalosis leads to a leftward shift in the oxyhemoglobin desaturation curve causing an increase in arterial oxygen saturation as a result of haemoglobin's greater affinity for oxygen and thereby increasing oxygen binding at the lungs (West, 2005). Under conditions of isocapnic hypoxia reducing SpO_2 to 85%, Minson and colleagues reported an increase in skin blood flow independent of changes in breathing rate (Minson et al., 1999). Sagawa et al., (1996) observed a doubling in forearm blood flow during acute exposes to an altitude of 5600m, and others have also reported increased T_{skin} at a given ambient temperature, suggesting a reduction in peripheral vasoconstriction by hypoxia (Blatteis and Lutherer, 1976; Cipriano and Golman, 1975). Resting $\dot{Q}$ increases by a magnitude dependant on the level of hypoxia employed. For example, when exposed to an F_IO_2 of 0.12, decreasing the arterial PO_2 to 40 mmHg, $\dot{Q}$ increases by 22%. As with heat stress, this increase in $\dot{Q}$ was mediated almost entirely by an increased heart rate, with SV maintained (Mazzeo, 2008). Oxygen delivery to the tissues is therefore tightly matched by immediate $\dot{Q}$ changes to peripheral demand in healthy, resting volunteers.

In summary, reduced PO_2 is sensed by chemoreceptors, which lead to an almost immediate increase in pulmonary ventilation, and subsequent hypocapnia and increases in pH, shifting the oxygen dissociation curve to the left. Peripheral vessels dilate in response to low oxygen, whereas vessels in the pulmonary vasculature constrict, shunting blood away from poorly ventilated regions, and thereby matching ventilation to perfusion. Cardiac output increases, mediated by an increased heart rate, while SV remains relatively stable.

2.1.4 Common cardiorespiratory responses to heat and hypoxia while resting

The common, shared cardiorespiratory responses to an acute resting hyperthermic or hypoxic challenge are summarised in Figure 2.1.

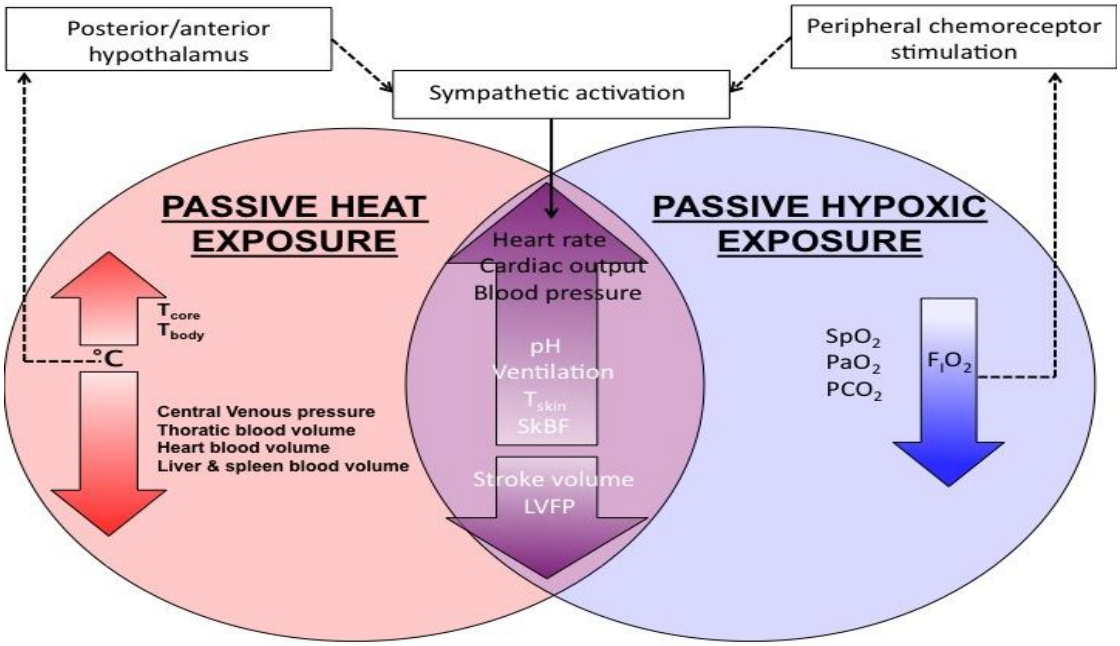

Figure 2.1. The common and different responses to passive heat or hypoxic exposure in humans. The red circle represents responses unique to heat stress, the blue circle represents responses unique to hypoxic stress, and the area of convergence represents the shared resting response between heat and hypoxia. The direction of the coloured arrow gradients indicate that as the magnitude of the stressor increases, the magnitude of the physiological responses increase accordingly.

2.2 Cardiorespiratory responses to heat and exercise stress

During exercise a transient increase in T_{core} occurs until an autonomically derived heat balance occurs, usually within 20 minutes of initiation of exercise (Webb et al., 1995). This plateauing of T_{core} during exercise has been observed over a large range of ambient temperatures (5 – 30°C) (Neilson and Neilson, 1962). At a fixed workload, the rise and

subsequent plateau in T_{core} is a function of relative exercise intensity, which dictates metabolic heat production, and relative exercise intensity, which modulates heat dissipation (Gonzalez-Alonso, 2008).

As with resting conditions, the rise in T_{core} during exercise in the heat has been consistently shown to increase ventilation during exercise when compared to normothermic conditions (Beaudin et al., 2009; Fujii et al., 2008, 2012; Hayashi et al., 2006, 2011; Nybo and Nielson, 2001). T_{core} has been identified as the primary stimulus for hyperthermic hyperventilation during exercise (Hayashi et al., 2006; Tsuji et al., 2012). The attenuation or prevention of exercise-induced increases in T_{core} has been shown to suppress pulmonary ventilation and to reduce the fall in $PaCO_2$ (Tsuiji et al., 2010, 2011). Exercise at a constant load of 57% $\dot{V}O_2$ max whilst under hyperthermic conditions (40°C) induced a 40% increase in exercise ventilation compared to the normothermic condition (18°C) (Nybo and Nielson, 2001). An increase in T_{core} can induce a thermal hyperpnea pattern of ventilation. This change in breathing pattern results in a hypocapnia and subsequent respiratory alkalosis (House et al., 1992; Cabanac and White, 1995). It is possible that the chemosensors also act as thermosensors. Indeed, the carotid bodies have been shown to have high Q_{10} rates of ≈75 *in vitro* (Gallego et al., 1979) and ≈3 *in-vivo* (Eyzaguirre and Lewin, 1984). Thus, temperatures would also affect their rate of discharge and presumably lead to proportionate increases in pulmonary ventilation (White, 2006). In support of this, chemosensor activity has been shown to respond in a similar manner to temperature sensitive neurons following a step-change in temperature, from 38°C to 40°C and then 42°C (Eyzaguirre and Lewin, 1984). This increase in ventilation as a response to heat and exercise has been suggested to aid heat loss from the upper-airways (Mariak et al., 1999). This may be an important heat loss avenue for human cranial thermoregulation. In support of this, small increases in upper airway

ventilation have been shown to produce local brain cooling in humans (Mariak et al., 1999). Thus the physiological function of thermal hyperpnea may be to act as a further heat loss mechanism (White, 2006).

The combination of exercise and hyperthermia has been described as the greatest challenge that can be imposed on the human cardiovascular system (Rowell, 1974). The cardiovascular response to heat is dictated by the magnitude of the thermal stress, and the duration and intensity of the work bout (Crandell and Gonzalez-Alonso, 2010). Occurring in parallel with the increase in T_{core}, heart rate increases, and stroke volume decreases over the course of prolonged exercise, termed cardiac drift (Coyle and Gonzalez-Alonso, 2001). Thus, for a given submaximal workload, HR is higher in hot conditions compared to cooler conditions.

Early research reported that forearm blood flow increased progressively during leg exercise in the heat (Johnson and Rowell, 1975). This lead to the later conclusions that exercise and heat produce a "competition" between the exercising skeletal muscle and the skin compartments for the available cardiac output. Thus blood flow to the active musculature would be decreased in order to increase perfusion at the skin to aid with heat dissipation (Rowell, 1978; Rowell, 1985). However when this was later tested, blood flow to both skin and muscle was adequately maintained during submaximal exercise in the heat (Laughlin and Armstrong, 1983; Armstrong et al., 1987; Savard et al., 1988; McKirnan et al., 1989; Neilson et al., 1990, 1993, 1997). The elevated demand being provided for by an increase in cardiac output, alongside vasoconstriction at splanchnic and renal circulations (Rowell, 1974).

Aerobic exercise performance is reduced in conditions of heat stress, and the risk of a heat related injury is increased. It is well established that maximum aerobic capacity ($\dot{V}O_2$ max) is

reduced when performed in hot conditions compared with thermoneutral environments (Bassett and Howely, 1999). The extent of the reduction appears to be proportional to the changes in body temperature. When T_{skin} is elevated, $\dot{V}O_2$ max is only marginally affected. However, the elevation of both T_{skin} and T_{core} magnifies reductions in $\dot{V}O_2$ max (Arngrimsson et al., 2003, 2004).

Data have shown that the magnitude in $\dot{V}O_2$ max reduction is greater in trained individuals compared to untrained counterparts. However this has not been investigated within the literature using different, distinct groups of fitness levels within the same investigation. An early study examining the effects of heat acclimation (HA) on aerobic fitness levels concluded that the reductions in $\dot{V}O_2$ max observed in the heat were not related to acclimation status or aerobic fitness (Sawka et al., 1985). However the small sample (n = 13) and small range of $\dot{V}O_2$ max values reported limits the application of these findings. As a consequence of the reduced aerobic capacity seen in the heat, studies have reported a reduced time to fatigue (Galloway and Maughn, 1997; Parkin et al., 1999) and reduced 30-minute time trial performance (Tatterson et al., 2000). Marathon times have been shown to be reduced by 2-3% in wet bulb globe temperatures (WBGT) > 20°C (Ely et al., 2007; Ely et al., 2008; Vihma et al., 2010; Wegeline and Hoffman, 2011). Multiple factors contribute towards this reduced capacity in the heat.

Table 2.1 displays a small subset of studies that have measured maximal aerobic capacity in both thermoneutral and hot conditions. Data from non-heat acclimated participants with a similar range of aerobic fitness values to those used in this thesis (Lee et al., 2013; 40°C,

20%RH) indicate that trained participants experience a greater magnitude in $\dot{V}O_{2\ max}$ reduction compared to untrained participants (Lorenzo et al., 2010; Lorenzo et al., 2011).

Table 2.1. Observed reductions in maximal aerobic capacity between thermoneutral and hot conditions. Data are mean ± SD.

Author	Thermoneutral $\dot{V}O_{2\ max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	Hyperthermic $\dot{V}O_{2\ max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	% Reduction in $\dot{V}O_{2\ max}$
Pirney et al., (1970)			7
Pirney et al., (1970)			20
Rowell et al., (1974)			3
Saltin et al., (1980)			8
Lorenzo et al., (2010)	66.9 ± 2.1	55.1 ± 2.4	18
Lorenzo et al., (2010)	70.2 ± 2.4	59.6 ± 2.0	15
Lorenzo et al., (2010)	66.8 ± 1.7	54.3 ±2.4	19
Lorenzo et al., (2010)	66.0 ± 2.4	54.9 ± 2.3	17
Lorenzo et al., (2010)	65.7 ± 2.0	54.9 ± 2.2	16
Lee et al., (2013)	45.1 ± 3	43.1 ± 2.0	7

2.2.1 Metabolic responses to exercise heat stress

Fink et al., (1975) were the first to show that environmental temperature affects intramuscular substrate utilization during submaximal exercise. During 60 minutes of intermittent exercise in ~ 41°C they reported an increase in intramuscular glycogen use and a decrease in triglyceride use versus exercise in cool conditions (9°C). Later, Kozlowski et al., (1985) studied muscle metabolism in dogs that ran for 57 minutes in temperate conditions (T_{core} 41.8 ± 0.2°C; muscle temperature T_{mu} 43 ± 0.2°C). The increase in T_{core} and T_{mu} were reduced by

45% through ice cooling during the run (T_{core} and T_{mu} 1.1 ± 0.2 and 1.2 ± 0.2°C respectively). An observed decrease, in hot conditions, in muscle ATP, PCr and glycogen and augmented increases in pyruvate, AMP and lactate led to the conclusion that hyperthermia accelerated fatigue by increasing the glycolytic rate and decreasing the energy potential from high energy phosphates (Kozlowski et al., 1985).

The same year, King et al., (1985) observed that prior to a period of heat acclimation, muscle glycogen content decreased more during a 6 hour walking test in humans when under heat stress. The authors hypothesised that this was a result of increasing circulating catecholamine concentrations acting upon phosphorylase activity. Neilson et al., (1990) found nor-adrenaline concentration increased whilst walking, and Febbraio et al., (1994) reported increased adrenaline concentrations during 40 minutes of cycling under heat stress, with both investigations observing concomitant reductions in muscle glycogen, thus confirming King et al's earlier hypothesis.

Febbraio et al., (1994) had 13 males complete two 40 minute cycling bouts at 70% $\dot{V}O_{2\ max}$, at 20°C, 20%RH or 40°C, 20%RH separated by one week. Hereafter the subjects underwent a 7 day heat acclimation period (90 minutes cycling at 50% $\dot{V}O_{2\ max}$) before completing another 40 minutes of cycling in 40°C, 20%. As well as the expected physiological responses to the heat stress trial (increased heart rate and core temperatures at all time points compared to thermoneutral), the authors also reported higher blood glucose and lactate values at all points compared to exercise at 20°C. Furthermore, post exercise muscle lactate values were increased in the pre acclimation heat trial, as was muscle glycogenesis and adrenaline, when compared with the control condition. Also observed was the preferential recruitment of Type I fibres as these were more glycogen depleted, suggesting no change in motor unit

recruitment in favour of fast twitch fibres. Increased blood glucose was observed which may be suggestive that elevated catecholamine concentrations also stimulate hepatic glucose release. This was later confirmed, although whole body glucose utilisation does not differ during submaximal exercise (Hargreaves et al., 1996).

Adrenaline plays an important role in the regulation of carbohydrate metabolism during prolonged exercise. It has been shown to increase the glycogenolytic rate of type I but not type II fibres, and that heat augments the use of glycogen in type I fibres alone (Greenhaff et al., 1991). Febbraio et al., (1998) infused adrenaline into subjects at 20°C in order to mimic the sympathetic-adrenal response observed at 40°C. The results showed that adrenaline does increase muscle glycogen use in males when cycling at 70% $\dot{V}O_{2\ max}$. It is worth noting that this level of exercise has been shown to have no effect on the energy charge potential or the ATP/ADP ratio in contracting muscle, irrespective of ambient temperature (Febbraio et al., 1994). This suggests that the increase in muscle glycogen use that occurs during heat stress is, in part, mediated by the enhanced sympatho-adrenal response (Febbraio et al., 1998).

Other studies have not reported a temperature-induced alteration in carbohydrate metabolism (Young et al., 1985; Nielson et al., 1990; Young et al., 1995; Maxwell et al., 1999). However, exercise itself leads to a degree of hyperthermia, thus the difference in core temperature when comparing experimental conditions must be of sufficient magnitude to induce altered substrate use. For example, Yaspelkis et al., (1995) reported no difference in glycogenesis when comparing exercise in the heat to cooler conditions. However, the largest T_{core} difference at any point between the two trials was 0.4°C. Another further confounding factor is the pre exercise glycogen level, as the rate of glycongenesis depends on this (Rowell et al., 1968; Chelsey et al., 1995). In the studies by Neilson et al., (1990), and Young et al., (1995), the pre exercise glycogen levels were higher prior to exercise in the cooler relative to warmer

conditions. Young et al., (1995) utilised a hot water training group and a cold water training group, each completing 60 minutes of underwater cycling at 60% $\dot{V}O_{2\ max}$, before and after training. In this study, pre exercise glycogen content was higher in pre exercise in the cold-water group leading to more glycogen being used in this trial. However, in the hot water training group before training (when pre exercise glycogen content was similar when comparing exercise in the hot with that in the cold water), the glycogenolytic rate during exercise was approximately 25% higher in the hot trial.

During exercise, muscle temperature (T_{mu}) rises in response to the increase in workload. This rise is augmented during exercise and heat stress. The rise in T_{mu} may act on key enzymes, which then alter exercise metabolism. Typical Q_{10} values for enzyme-mediated reactions are 2.0 to 3.0. So, for every 10°C rise in temperature a 2-3 fold increase in enzyme reaction rate is observed (Florkin and Stiltz, 1968). Thus, the 2°C increase in T_{mu} observed during exercise and heat stress could feasibly increase enzyme rates by 20-30%. The role the Q_{10} effect plays on muscle metabolism during heat stress was investigated by Febbraio's group using heat pads or water perfused cuffs to manipulate T_{mu}. Febbraio et al., (1996) elevated T_{mu} before and after intense or submaximal (Starkie et al., 1999) exercise. They found that elevated T_{mu} increased the rate of glycogenolysis and lactate accumulation in the absence of changes in body temperature or plasma catecholamine levels during 2 minutes of cycling at 115% $\dot{V}O_{2\ max}$. Also observed was an augmented decline in the TAN pool and an increase in IMP accumulation.

It is possible that this increase in glycongenolysis and anaerobic glycolysis resulted from a Q_{10} effect on glycogenolytic and glycolytic processes. Another explanation is that the decrease in TAN modulated these alterations. For example, TAN degradation (mainly

increases in free ADP) leads to allostatic activation of the glycogenolytic and glycolytic enzymes phosphofructokinase (PFK) (Uyeda, 1979) and phosphorylase (Ren and Hultman, 1990). Starkie et al., (1999) heated one leg and cooled the other for 40 minutes before and 20 minutes during cycling at 70% $\dot{V}O_{2\ peak}$ using water perfused cuffs. A 6.9 + 0.9 °C difference in T_{mu} between the heated and cooled leg was observed after the 40-minute pre exercise treatment. This difference was reduced during the 20 minute cycling bout, but was still significantly different upon termination of exercise. In the heated leg, an augmented rate of glycogen use but no difference in high-energy phosphogen metabolism was noted, when compared with the cooled leg. This suggests that temperature *per se* plays a regulatory role in intramuscular carbohydrate utilization and appears to be responsible, in part, for the frequently documented increase in glycogen utilization during exercise and heat stress.

It can be concluded that if submaximal exercise in the heat results in a marked increase in core body temperature (>0.5°C), then intramuscular carbohydrate utilization in augmented. If, however, the body temperature is not markedly influenced by exercise and heat stress, it is unlikely that metabolic differences will be observed (Febbraio, 2001). When the rise in core temperature was attenuated by a 7-day heat acclimation period, muscle glycogenesis was reduced (Febbraio et al., 1994). The same effect is also seen by preventing dehydration (Gonzalez-Alonso et al., 1999), cooling (Febbraio et al., 1996), or reducing ambient temperature (Kozlowski et al., 1985).

2.2.2 Cardiorespiratory responses to hypoxia and exercise stress

As with the resting hypoxic response, exercise also increases minute ventilation for a given workload when compared to sea level conditions (West, 1982). During maximal intensities of exercise at altitudes of 6400m, values in excess of 200L•min^{-1} (BTPS) have been reported

(Pugh et al., 1964). Altitude exposure is associated with a decrease in aerobic capacity that parallels the decrease in barometric pressure or inspired PO_2 and is thus explained primarily by the decreased O_2 saturation of the blood (Cerretelli et al., 1980). A larger decrease in $\dot{V}O_{2\ max}$ in trained versus untrained individuals has been reported in both rats (Favret et al., 2003, 2006) and humans (Martin and O'Kroy, 1993; Woorons et al., 2005; Mollard et al, 2007). The difference has been attributed to the larger arterial desaturation during exercise in hypoxia, because of the greater cardiac output and faster pulmonary transit time, and a greater O_2 saturation by the muscles in trained volunteers. This would lead to a lower PO_2 in the mixed venous return to the lungs.

The intensity of exercise and relative stress of the work bout plays a major role in the subsequent physiological responses to hypoxia. In comparison to the same absolute workload at sea level, greater relative work intensity is achieved in hypoxic conditions, accompanied by a greater homeostatic disruption. Therefore greater physiological and metabolic adjustment is required when performing identical tasks in hypoxic versus sea-level conditions (Mazzeo, 2008). For example, $\dot{V}O_{2\ max}$ at an elevation of 4300m was 25% less than recorded at sea level. Thus when participants cycled at 100Watts, eliciting a $\dot{V}O_2$ of 1.5L•min^{-1}, this represented a relative workload of 50% normoxic $\dot{V}O_{2\ max}$. When the same workload (100W) was performed at 4300m, an identical $\dot{V}O_2$ of 1.5L•min^{-1} was reported, however this represented 65% of hypoxic $\dot{V}O_{2\ max}$ (Mazzeo et al., 1991; Mazzeo et al., 1995; Wolfel et al., 1991; Wolfel et al., 1998). The reduction in $\dot{V}O_{2\ max}$ observed under hypoxic conditions is due to 4 main factors: the reductions in inspired PO_2 and impairments of gas exchange induced arterial desaturation; the reduction in maximal cardiac output; and the limitation of diffusion. The relative importance of each is dependent on the severity of hypoxia. The rate

of $\dot{V}O_2$ is a function of blood flow (cardiac output) and tissue utilization [(a-$\bar{v}$)-O_2] difference. This is best illustrated by the Fick equation:

$$\dot{V}O_2 = \dot{Q} \times (a\text{-}\bar{v})\ O_2 \text{ difference.}$$

An examination of the components that make up the Fick equation allows for the elements associated with oxygen delivery to be evaluated in reference to hypoxia.

Cardiac output is increased during exercise in order to compensate for the decline in SaO_2; this ensures adequate delivery of O_2 to the tissues. At submaximal workloads an increased cardiac output and local muscle blood flow provide sufficient O_2 to the muscles. Thus for a given workload, $\dot{V}O_2$ is the same as would be observed at sea-level (Calbet et al., 2003). The increase in $\dot{Q}$ is mediated by an increased HR. The increased HR is due to cardiac sympathetic nerves stimulating the cardiac β-adrenogenic receptors, and increases in circulating adrenaline. During submaximal exercise SV remains unaffected. However as work reaches maximal intensities, O_2 delivery cannot keep pace with demand, creating a mismatch, as noted by the steady decline in $\dot{V}O_{2\ max}$ with increasing altitude (Grover et al., 1986; Calbet et al., 2003; Lundby et al., 2006). Thus reductions in $\dot{V}O_{2\ max}$ during acute exposures to altitude are due primarily to the decline in arterial oxygen content (CaO_2) (Wolfel et al., 1991; Grover et al., 1986).

The rate of O_2 delivery and local blood flow contribute to O_2 utilization by working muscle, and diffusion of O_2 from the capillary into the muscle are each factors determining the a-$\dot{V}O_2$ difference. The mitochondrial oxidative capacity of muscle cells, and the metabolic demand/rate placed upon the cell are perhaps the main factors determining the a-$\bar{v}O_2$ difference (Mazzeo, 2008). During studies at altitudes of 4300 meters, blood flow during acute submaximal exercise remains largely unchanged from sea-level (Wolfel et al., 1991;

Wolfel et al., 1998). Findings later confirmed at higher altitudes (5260m, Cornolo et al., 2004). That exercising muscle extracts almost identical O_2 before and after acclimatization would suggest that O_2 diffusion capacity is not limiting at altitude (Lundby et a., 2006). Thus as metabolic rate increases during submaximal exercise O_2 extraction is maintained.

2.2.3 Metabolic responses to hypoxia

In oxidative tissues, production of cellular ATP occurs via oxidative phosphorylation at the inner mitochondrial membrane. Thus, in order to sustain normal cellular function, the mitochondria require a constant supply of fuel and oxygen. This is illustrated in Figure 2.2.

When presented with a hypoxic challenge, adjustments to cellular energy homeostasis must occur. A greater contribution of anaerobic glycolysis to ATP production occurs, particularly during exercise at altitude (Mazzeo, 2008). When assessing alterations in substrate use under hypoxic conditions, care must be taken when comparing values to those obtained during normoxic submaximal exercise. As previously outlined, as $\dot{V}O_{2\ max}$ decreases with increasing altitudes, a similar absolute workload performed at sea level represents a greater relative workload when performed at altitude. Thus as a reliance on carbohydrates as fuel increases with increasing exercise intensity, comparisons with the same absolute workload would confound data interpretation.

Figure 2.2. Mitochondrial energy metabolism. Fatty acid oxidation and the TCA cycle produce NADH and $FADH_2$, which are oxidized by complexes 1 and II of the electron transport chain. Electrons are transferred through the chain to the final acceptor, O_2. The free energy from the electron transfer is used to pump H^+ out of the mitochondria and generate an electrochemical gradient across the inner mitochondrial membrane. This gradient leads to the synthesis of ATP via ATP synthase. Taken from Murray, (2009).

Short-term exposures to high altitude results in an increase in blood glucose utilization when compared to the same absolute workload at sea level, and this persists until at least 21 days of acclimatization (Brooks et al., 1992; Roberts et al., 1996). This shift in substrate utilization may be advantageous given the lower oxygen cost of metabolising glucose when compared with fats and proteins (Martin et al., 2010), thereby representing a more efficient use of fuels. However, no differences in substrate utilization were observed when work rate was matched to the same relative exercise intensity at sea level (Lundby et al., 2002). In addition to this enhanced capacity for glycolysis, an active shunting of pyruvate, the end product of glycolysis, towards lactate production and away from oxidative metabolism in the mitochondria is upregulated in hypoxia (Semenza et al., 1994). The exclusion of pyruvate from mitochondrial oxidation occurs alongside an upregulation in lactate dehydrogenase, which converts pyruvate to lactate. The transport of lactate out of the cell is mediated by monocarboxylate transporters 1 and 4 (MCT1 and MCT4). MCT4 but not MCT1 have been found to be upregulated under conditions of hypoxia in cell lines (Ullah et al., 2006). However, when examined *in vivo* in humans the results have been less conclusive. For example, although skeletal muscle MCT1 and MCT4 were unaltered following acclimatization to 4100m, acute exercise in the same level of hypoxia induced increases in MCT1 mRNA (Zoll et al., 2006). This may suggest that glycolytically derived lactate is expelled more readily from the hypoxic cell (Simon et al., 2006).

During exercise in acute hypoxia blood lactate for a given level of exercise is greater when compared to the same absolute workload at sea level, as a result of the change in relative work intensity. However, after a period of acclimation, exercise at the same absolute workloads performed prior to the adaptive period, and also at maximal workloads, results in lower levels of lactate (Green et al., 1992). This has been termed the lactate-paradox,

although its existence is still open to debate (van Hall, 2007). Providing it exists, this phenomenon may relate to alterations in the coupling of glycolysis and oxidative phosphorylation, with rising ATP/ADP ratios inhibiting glycolysis, and consequently reducing lactate production (Bartlett and Leonard, 2010).

2.2.4 Common responses between heat and hypoxic exercise

Positive cross-acclimations have been suggested to occur when stressors arouse the same, or many similar physiologic strains (Fregly, 1996). The sections above identify many overlapping physiological strains elicited from acute individual exposures to either heat or hypoxia. Though driven by a drive to increase heat loss and restore thermal balance in heat, and to increase oxygen delivery in hypoxia, many convergent responses to these homeostatic perturbations indicate a potential for cross-acclimatory effects at the whole body level, and are summarised in Figure 2.3.

Interestingly, to date only 1 study, to the authors' knowledge, has examined the combined physiological stress of heat and hypoxia in humans (Hale, 1960). In this brief abstract, pilots were exposed to a combination of 49°C, 20%RH, and an altitude equivalent to 5485 meters above sea level, at rest. The addition of heat augmented the cardio-accelerative response to hypoxia in 12 out of 19 participants, whereas 7 displayed bradycardia and syncope. Also noted was a progressive decline in systolic blood pressure in all participants (Hale, 1960). This lack of research into the combined effects of these environmental stressors is surprising given the nature of current military operations, which often sequentially expose troops to conditions of high temperature with later operations at high altitude (Rodway and Muza, 2011). During a 130 minute foot patrol in which the ambient temperature ranged 39-47°C mean core temperatures of 38.8 ± 0.5°C and mean heart rates of 150 ± 5 beats•min^{-1} have

been reported (Buller et al., 2008). In addition, the mountainous regions of Afghanistan, which can be as high as 3000m can also have temperatures as high as 27°C (Nindl et al., 2013). Studying the individual and combined responses to heat and hypoxic stress within the same group of participants has received little attention within the literature and would therefore be of interest. This would provide a useful starting point when examining potential cross-acclimation effects between stressors (Fregly, 1996; Tipton, 2012). The physiological responses to repeated exposures to heat and hypoxia are discussed in Sections 2.3.1and 2.3.2.

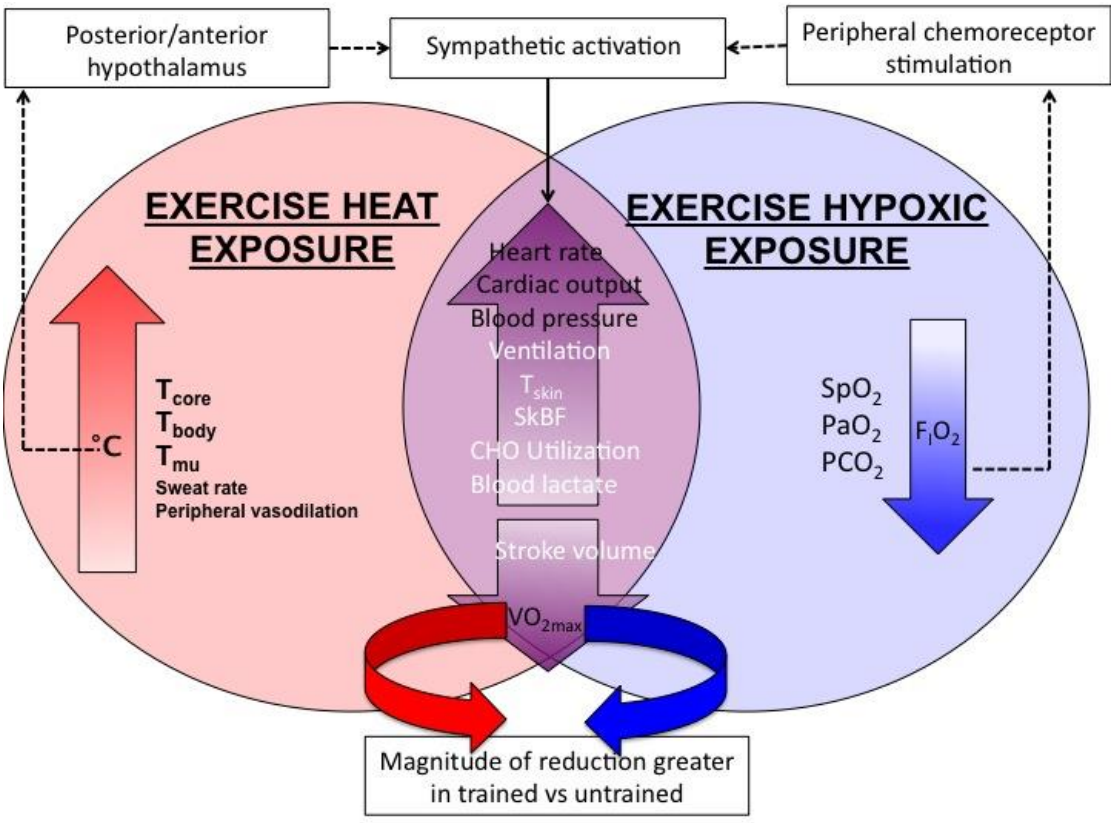

Figure 2.3. The shared and different response to acute submaximal exercise in heat or hypoxia. The red circle represents responses unique to heat stress, the blue circle represents responses unique to hypoxia, and the area of convergence represents the shared physiological responses between these stressors.

2.3 Adaptive responses to heat and hypoxia

Adaptation is one of physiology's fundamental tenets, operating not only at the level of the species, but also at the level of the tissues, cells, molecules and genes. The ability of a stressor to induce adaptation is a function of its intensity, duration and frequency, and the genetic and phenotypic status of the individual (Adolph, 1964). Bannister et al., (1980) adapted the training impulse model to quantify the cumulative adaptation impulse (stress volume). For example, a thermal impulse can be calculated from the sum changes in body temperature, and the duration spent under the adaptive stimulus. This represents a simple and useful means of comparing adaptation stimuli (Taylor and Cotter, 2006). Physiological systems contain many common and discreet effector organs, and the nature of systemic and organ adaptations can vary greatly across stimuli, or within an adaptation stimulus (Taylor and Cotter, 2006). Thus adaptation is an integrated physiological response that affects many structures and mechanisms simultaneously, and at different rates (as represented by short term and long term heat acclimation, Section 2.3.). Figure 2.4 illustrates the conceptual time course of adaptation (Adolph, 1964).

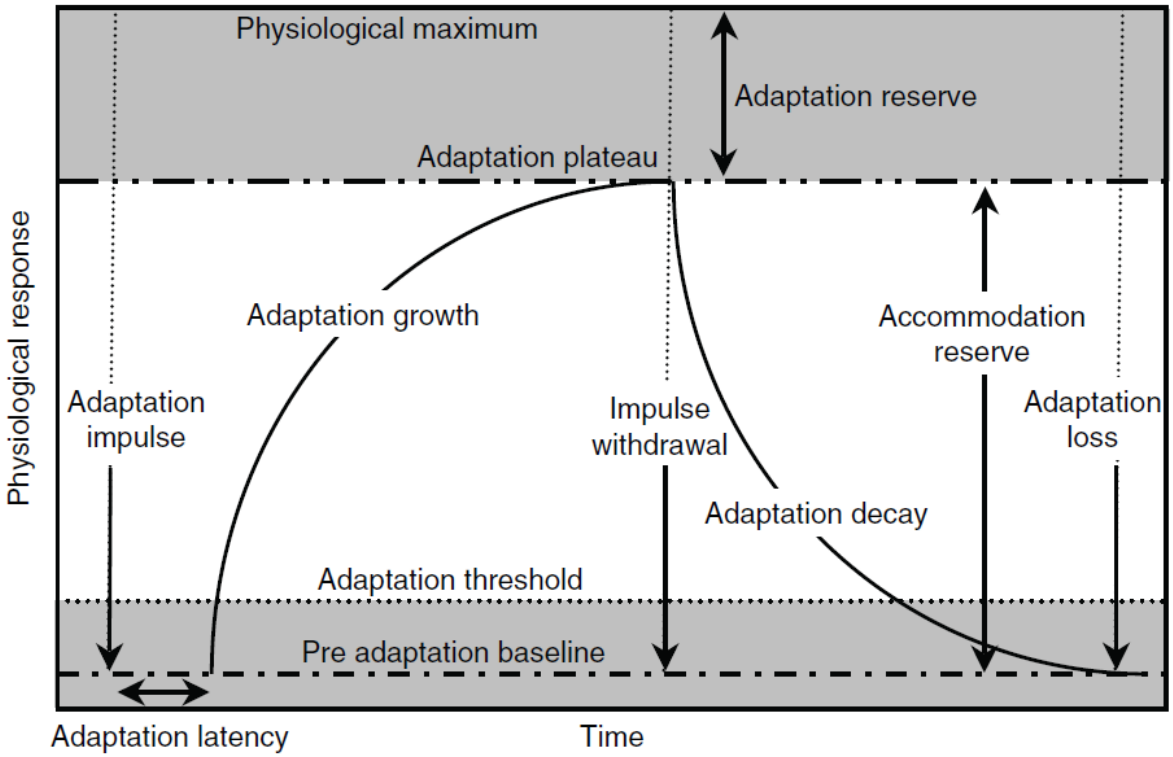

Figure 2.4. Adaptation theory. This illustrates a hypothetical scenario in which physiological changes occur following the application and also after the later withdrawal of a stressor (Adolph, 1964). With the application of a sufficiently strong stimulus homeostasis is disturbed. If this disruption is large enough, the adaptation threshold will be exceeded and physiological adaptation will be initiated, albeit with some delay (latency). Adaptation impulses that do not induce systemic failure will eventually elicit more complete adaptation (plateau), with the difference between the genetically determined physiological maximum and the adaptation plateau defining the potential for further improvements to be realized (adaptation reserve). Variations in the location of this plateau reflect changes in the capacity to tolerate stress (accommodation reserve). Finally, removal of the thermal impulse for a sufficiently long duration results in a decay of these acquired physiological adaptations (Taylor, 2011).

2.3.1 Heat acclimation

For the purposes of this thesis, acclimation refers to an artificially induced adaptation to a stressor minus other climatic components (i.e. as occurs in a laboratory setting; Aoyagi et al., 1997) and the literature reviewed is primarily human based. Heat acclimation consists of an array of autonomically controlled physiological processes, which work in concert to improve heat dissipation, and delay thermal injury (Horowitz, 1998). Acclimation is modified through integrated autonomic, cellular and molecular responses, each of different intensities, time courses and direction.

The primary stimuli for heat acclimation are the sustained increases in T_{core} and sweating (Taylor et al., 2000). Thus while passive heat exposure and exercise in a temperate environment offer some benefit (Gisolfi and Robinson, 1969), exercise within the heat represents the most effective means of adaptation due to the exogenous (ambient) and endogenous (metabolic) heat loads (Nadel et al., 1979). Human acclimation to the heat is rapid, with 80% of the physiological adjustments achieved within the first 7 days of exposure, although the physiological responses before and after acclimation depend on the length and intensity of the exposure (Robinson et al., 1943; Fox et al., 1963; Fox et al., 1964). Heat acclimation primarily results in an increased capacity of thermal effectors for heat dissipation, decreased heat production, and an expanding body core safety margin. These adaptations are manifested as a reduction in resting T_{core} and a reduced T_{core} for a given level of exercise, creating the greater cooling capacity during the exercise bout (Brooks et al., 2005; Fink et al., 1975; Gisolfi and Robinson, 1969; Nadel et al., 1979; Neilson et al., 1993; Neilson et al., 1997). The greater cardiovascular stability associated with heat acclimation (i.e. lower exercising heart rates mediated by an increased stroke volume) are thought to be due to hypervolemia and plasma volume expansion due to increases in plasma proteins and

sodium and chloride retention (Neilson et al., 1993; Nielson et al, 1997). These cardiovascular adjustments occur earlier during the acclimatory cascade of events (< 7 days; Garrett et al., 2011). A lower T_{core} threshold for the initiation of vasodilation and increased skin blood flow facilitate a more rapid transference of heat from the body core and active muscle to the periphery, thereby improving evaporative heat loss. Longer-term adaptive regimes increase the volume and diluteness of sweat, reducing T_{skin} further.

2.3.1.2 Plasma volume expansion

It is now broadly accepted that a heat induced increase in the PV is necessary to reduce CV strain during subsequent heat stress. PV expansion also increases the specific heat of the blood, allowing for greater heat transfer at the skin (Sawka, 2011). Under normal resting conditions the Starling forces (hydrostatic and oncotic forces in the movement of fluid across capillary membranes) favour filtration at the alveolar ends of capillaries and flid reabsorption at the venous ends (Taylor, 2014). During exercise in the heat, a blood flow redistribution into the exercising skeletal muscle increases the intravascular pressure of the capilleries (Bjornberg, 1990), causing a fluid shift into the interstital compartment (Morimoto et al., 1978). During heat acclimation, participants will repeatedly undergo these acute intravascular shifts, eventually resulting in a blood volume enlargement (Senay, 1979; Maw et al., 1996). During heat acclimation, this is mostly due to the combined effects of elevated plasma colloid and crystalloid osmotic pressures altering the Starling forces (Wyndam et al., 1968). Under such circumstances Starling forces favour absorption, and result in an intravascular influx from the interstitial compartment (Taylor, 2014). This fluid can be retained, depending on the duration, magnitude and frequency of the adaptive stimulus.

When adapting to heat loads, both the cutaneous blood vessels and the eccrine sweat glands are sequentially activated, with elevated cutaneous blood flow required to transport heat to

the periphery (Sundstroem, 1927). Three classical sudomotor adaptions to heat have been described (Wyndham et al., 1968): a reduced core temperature threshold for the onset of sweating; an increased sensitivity of sweat secretion to changes in core temperature; an elevated capacity for sweat production at an equivalent core temperature (Wyndham et al., 1968). The composition of sweat varies across skin regions, and sodium can be actively and selectively reabsorbed within the distal duct of a sweat gland, while chloride absorption is passive (Sato, 1977). Heat acclimation has been shown to elevate electrolyte reabsorption and reduce sodium secretion (Bass et al., 1955; Bass and Henschal, 1956; Allan and Wilson, 1971).

The compositional change in sweat enhances evaporative cooling because electrolytes lower the cutaneous water vapour pressure for a given skin temperature. Thus the more dilute the sweat secreted post heat acclimation is more readily evaporated. The effect of this is not only to conserve salt, but also water as the enhanced evaporation defends body temperature without the requirement for greater secretion (Taylor, 2014). This is another example of a physiological system moving from an early inefficient state to a greater level of efficiency later on in the adaptive continuum. The sodium conserving effect of heat acclimation depends on the adrenal cortex; and the hormone aldosterone appears necessary for its occurrence (Sawka et al., 1996). Increased plasma aldosterone concentrations are observed early on in the acclimation process (Francesconi et al., 1983) and are associated with reduced sodium concentrations in sweat. When participants were injected with an aldosterone precursor (deoxycorticosterone acetate) sodium and chloride concentrations in the sweat are reduced and plasma volume and body mass increased (Ladell, 1945). That aldosterone induces greater sodium reabsorption from the sweat glands would indicate that it is an integral part of the

adaptation process, with greater sodium and chloride retention facilitating blood volume defence and even its elevation.

2.3.1.3 The biphasic nature of heat acclimation: short term and long term adaptations

In rodent models of heat acclimation, it has been demonstrated that variations in the temperature thresholds for the major heat dissipation mechanisms during the progression of acclimation occur in two distinct phases. Principally, the marked initial temperature drop, signifying an early autonomic response during short term heat acclimation (STHA) is associated with perturbed peripheral effector cellular performance (Horowitz et al., 1983; Kloog et al,. 1985). Pre-acclimation values return when acclimatory homeostasis is reached: long -term heat acclimation (LTHA; Horowitz et al., 2001, 2002).

In humans, Senay et al., (1976) provided substantial evidence that acclimation of different effectors is not simultaneous, and that adaptations of each effector sometimes involve an array of opposing processes. This has been extensively examined in intact-rodent models of heat-dissipation. For example, heat dissipation effectors have shown an apparent acclimated state soon after short periods of exposure, which is brought about by a cascade of transiently recruited mechanisms that alleviate strain. Animals undergoing heat treatment display transiently impaired effector responsiveness during the initial phase of heat acclimation. This is compensated for by an increase in autonomic activity. Over the time course of acclimation the short-term mechanisms were superseded by more efficient long-lasting mechanisms, which provide an optimal adaptive state. Thus acclimation can be thought of as a continuum of processes varying temporally and differing in efficiency and optimal performance (Horowitz, 1998).

In the initial STHA (1-5 days) the effector organ output to autonomic signal (EO/AS) ratio transiently decreases; i.e. accelerated efferent activity is required to override impaired peripheral responsiveness to produce the desired effector output. Thus, in this period the ANS is the major effector in alleviating strain (Horowitz et al., 1998). During the acute response to heat acclimation (days 1-5), the expansion in plasma volume improves the maintenance of the osmotic potential of the blood. This expanded vascular volume during the STHA phase allows a more effective regulation of blood pressure despite increased fluid loss during exercise heat stress. Thus for a given workload, stroke volume is increased, and the heart rate lowered. Towards the end of the STHA period, the amount of blood directed towards the skin is elevated, and the threshold for vasodilation lowered (Garrett et al., 2008; Patterson et al., 2005), which facilitates the loss of central heat. Because central blood volume and mean arterial pressure are better defended, the cardiovascular drift associated with endurance exercise under hot conditions is reduced. Thus by the end of the STHA phase cardiac efficiency is increased (Garrett., 2010).

Once acclimatory homeostasis has been achieved (> 3 weeks), intrinsic organ adaptations, such as increased heat shock protein 72 (HSP72) and heat shock protein 90 (HSP90) reserves (section 2.4), reduces the requirements of accelerated excitation, which manifests by an increased effector output despite decreased autonomic stimulation (an increase in EO/AS; Horowitz, 2007). A characterisation of this longer term exposure (7-14 days in humans) is the elevated sweat secretion. This occurs via the increased sudomotor sensitivity, and the reduced threshold for the onset of sweating. This two-phase response of heat acclimation is illustrated in Figure 2.5.

Figure 2.5 A conceptual model of heat acclimation. During STHA accelerator autonomic discharges override the impaired effector organ (EO) responsiveness. During phase 2 of heat acclimation (LTHA), the share of peripheral cellular adaptive features in homeostasis control increase under environmental constraint, diminishing the accelerated autonomic discharge. Other central changes, such as decreased temperature thresholds for the activation of heat dissipation effectors with concomitant increases in their sensitivity occur in this phase. Taken from Horowitz, (1998).

2.3.1.4 Methods of heat acclimation

Heat acclimation methods can be grouped into 3 main types, 1) constant load exercise 2) self-regulated exercise, and 3) controlled hyperthermia (Taylor, 2000). The most commonly used method is the constant load technique, which requires participants to adhere to a set workload throughout the course of the acclimation period, e.g. a workload of 50% $\dot{V}O_{2\ max}$, 60 minutes

per day for 10 days (Castle et al., 2011). Self-paced regimens allow participants to regulate their own work rates based on their individual perceptions of thermal strain (Armstrong et al., 1986; Sandstrom et al., 2008). This technique is more suited to practical, rather than research applications (Garrett et al., 2011). The controlled hyperthermia technique ensures that all participants undergo a similar level of endogenous thermal strain as it involves elevating and maintaining a steady core body temperature above the sweating threshold using exercise. Body temperature may be elevated prior to exercise using passive means, such as pre-heating in a water bath, or through the use of a water perfused suit (Turk and Worsley, 1974). The goal of the exercise bout is to then complete a given level of work (e.g. 60 minutes) above a target temperature (e.g. 39.0°C, Magalhaes et al, 2010).

Other studies have used a higher workload for 30 minutes (60% - 75% $\dot{V}O_{2\ max}$) to raise core temperature, which is then maintained at the desired level by adjusting the work rate during the exercise session (Fox et al., 1963; Fox et al, 1964; Patterson et al., 2004). It is likely that this model offers a more complete adaptation to heat as the daily thermal strain (i.e. increase in T_{core}) is maintained throughout the acclimation period (Garrett et al., 2011). However the changes in exercise workload required to manipulate internal temperatures may vary between participants throughout the protocol. Plasma volume expansion, which is considered a transitory aspect of acclimation, has been shown to be elevated for at least 21 days when the controlled hyperthermia method was used (Garrett et al., 2008) However, few studies have compared the efficacy of different acclimation protocols (Taylor and Cotter, 2006).

Figure 2.6 demonstrates the differences between the constant load protocol and the controlled hyperthermia protocol of acclimation, each 90 minutes in duration and each aimed initially at increasing the T_{core} to >38.5°C. Constant load protocols produce a gradual rise to the target T_{core} (Figure 2.6, panel A), however adaptation throughout the acclimation period results in

the physiological impact of each session becoming progressively smaller, being reflected in the decreasing core temperature. This leads to a reduction in the adaptive stimulus upon repeat exposures, illustrated in the inset figure showing a fall in the cumulative core temperature. The controlled hyperthermia protocol (Figure 2.6, panel B) elicits a more rapid T_{core} rise, and it then held constant during the repeat exposures. This happens as work rate increases as adaptation progresses, resulting in an increasing adaptation stimulus, evidenced by the more stable cumulative core temperature index (Figure 2.6 panel B inset). Each of these methods elicit adaptation.

Whilst the controlled hyperthermia model undoubtedly places a greater level of strain on an individual, certain methodological features make this technique problematic. For example, studies using exercise to attain the desired T_{core} prior to the actual acclimation may lead to different levels of work being completed between participants, a factor that is methodologically challenging to control for. Depending on the research question being asked, this may make separating the effects of heat acclimation *per-se*, from exercise difficult.

Figure 2.6. Mean core temperature changes on days 1, 4, 8 and 12 of two, 12-day heat acclimation regimens using either the constant work rate (A) or controlled hyperthermia methods (B). Insets show integrated core temperature for each day (Taylor and Cotter, 2006).

Later in this thesis (Chapters 5 and 6), the effects of 3 and 10 days of heat acclimation on physiological tolerance to acute hypoxia was compared to the responses recorded using identical periods of work in either control or hypoxic conditions. In light of this it was reasoned that the use of a constant load heat acclimation protocol (60 minutes/day 50% $\dot{V}O_{2max}$) that has been shown to induce heat acclimation (Castle et al., 2011) would be a simple model to employ in these studies (Chapter 5 and 6).

2.3.2 Altitude acclimation

Over the first few days of residence at altitude, many physiological adaptations occur to improve overall tolerance. Two adaptations in particular reduce the level of hypoxemia potentially encountered: these being increased ventilation, and decreased plasma volume (hemoconcentration). Ventilatory acclimation occurs via a progressive increase in ventilation, PaO_2, and SaO_2, alongside a reduction in arterial $PaCO_2$ and normalisation of arterial pH. These responses typically occur between days 5 – 9 (Bisgard and Forster, 1996). A reduction in plasma volume alongside this increase in ventilation produces hemocentration (Hoyt and Honig, 1996). The net result of these adaptations, which can take up to 14 days of high altitude residence, is a normalisation in CaO_2.

After a few days at altitude, the increased resting $\dot{Q}$, which is mediated by the increase in HR returns to near normoxic values. Heart rate remains elevated as a result of the decline in SV. This is a consistent feature of hypoxic adaptation, with $\dot{Q}$ no different from sea level, but HR elevated and SV depressed (Hartley et al., 1967; Bandero et al., 1968; Vogel et al., 1974). That the increase in $\dot{Q}$ occurs before hemoconcentration indicates O_2 extraction may be enhanced. A 10-day residence at an altitude of 3100m showed a decrease in coronary blood flow compared with sea level, which was in proportion to the fall in left-ventricular work due

to the increase in O_2 content in arterial blood that occurs during acclimation. Thus myocardial O_2 extraction per volume of blood is increased to maintain myocardial oxygenation.

During exercise, $\dot{Q}$ is reduced at a given power output due to the depressed SV, which is likely caused by a fall in venous filling and a decrease in HR linked to depressed cardiac responsiveness to β-adrenogenic stimulation (Roach et al., 1999) and an increase in vagal tone (Hopkins et al., 2003; Cornolo et al., 2004). The fall in SV is associated with a reduction in left ventricular dimensions and filling pressure (Reeves et al., 1987), which may be mediated by diuresis and the decreasing plasma volume (Grover et al., 2001). The fall in PV may be caused by the chemoreceptors, an increase in atrial peptide, and a decreased synthesis of aldosterone (Swenson et al., 2001), which occurs over the first weeks of exposure. Later falls in PV occur without net loss of body water by fluid shifts from the extracellular to the intracellular compartments (Bartsch and Gibbs, 2007). Muscle blood flow has been shown to decrease following 21 days exposure to 4300m (Wolfel et al., 1991). That this occurs in conjunction with the increase in CaO_2, during submaximal exercise, O_2 delivery is regulated to match demand.

The elimination of CO_2 by the increases in ventilation decreases the hydrogen ion concentration in the blood, thereby producing respiratory alkalosis. This alkalosis affects the affinity of haemoglobin for O_2, making it easier for red blood cells to obtain O_2 from the lung capillaries. The effect of this is best observed in the Hb saturation curve, which shifts to the left, allowing the half-saturation of Hb at a lower PO_2. Thus, it is easier for Hb to pick up O_2, but harder for it to release O_2 to the tissues. A persistent alkalytic state results in the increase of 2,3 diphosphoglycerate (2,3 DPG) in the red blood cells. This allows for the easier release

of O_2 (Piantadosi, 2003). The hemodynamic effects of altitude acclimation are discussed in the next section.

2.3.2.2 Hemodynamic adaptation to hypoxia

Shortly after arriving at altitude, the kidneys compensate for the increased alkalosis, induced by the increased ventilation, by eliminating excess base from the blood, in the form of the bicarbonate anion (HCO_3^-). This shifts the O_2 dissociation curve back to its original position. During the first few days at altitude, the kidneys excrete salt and water, decreasing the plasma volume and allowing for an increase in hemoglobin. This increase in Hb and increase in the number of circulating red blood cells (RBC) is an important feature of altitude acclimation, and is the targeted aspect of altitude training to enhance sea level performance (Stray-Gunderson, et al., 1998).

The combined effects of erythrocytosis and a decreased PV are called Polycythemia (Jacobs et al., 2012). This can increase the O_2 carrying capacity of the blood by one-third, depending on the severity of altitude experienced and the individuals' adaptive capacity. Although the cardiorespiratory responses to altitude occur almost immediately upon exposure, the alterations in red cell mass and Hb concentrations occur more gradually as they require a change in gene expression (Piantadosi, 2003). The maximal concentrations of Hb develop over several weeks, as hypoxia first mediates a fall in plasma volume, and then an increase in red cell mass under the control of the renal hormone erythropoietin (EPO). EPO is largely produced in the cells of the kidney and is responsible for the regulation of red blood cell production (Wang and Semenza, 1996). The increase in EPO requires an optimal threshold of hypoxaemia. Ge et al., (2002) concluded that this threshold for EPO lies at altitudes of >2100

to 2500m. When the F_IO_2 is equivalent to 0.14 – 0.16, EPO concentrations are significantly increased.

Erythropoietin concentrations have been shown to increase as a result of 70 – 120 min of acute and continuous hypoxic exposure (Eckardt et al., 1989; Rodreiguez et al., 2000). Knaupp et al., (1992) demonstrated that at least 120 minutes of continuous hypoxia (F_IO_2 0.105) is required to augment kidney-induced EPO production. The hypoxia induced increases in EPO are ultimately governed by upstream physiological characteristics involved in the delivery of O_2 to renal tissue (Ge et al., 2002) and the transcription factors associated with the O_2-sensitve protein complex known as hypoxia-inducable factor-1 α (HIF-1 α) (Jedlickova et al., 2003).

2.3.2.3 HIF-1 α: Regulation and target genes

Hypoxia-inducible factor is expressed by all metazoan species analysed to date (Loenarz et al., 2012). HIF consists of two subunits, each containing a basic helix loop helix-PAS domains (Wang et al., 1995) that mediate heterodimerisation, with other BHLH-PAS proteins. These are present in excess, so that HIF-1 protein levels determine transcription levels (Semenza, 1996). Under normoxic conditions HIF1-α is bound by the Von-Hippel-Lindau (VHL) protein. VHL recruits an ubiquitin ligase that targets HIF1-α for proteosomal degradation (Kaelin and Ratclife, 2008). VHL binding is dependent on hydroxylation of a specific protein residue in HIF1-α by the prolylhydroxylase PHD2. PHD2 uses O_2 as a substrate and therefore its activity is inhibited in conditions of hypoxia (Epstein et al., 2001) and factor inhibiting HIF1 (FIH-1) represses HIF-1α transactivation factor (Malion et al., 2001). This hydroxylates an asparaginyl residue in HIF1-α, using O_2 and α-ketogluterate as substrates, blocking the association of HIF1-α with the P300 activator protein (Lando et al.,

2002). Diemethyloxalyglycine (DMOG), a competitive antagonist of α-ketongluterate, inhibits hydroxylases and induces HIF1 dependent transcription (Epstein et al., 2001).

Figure 2. Mechanisms of HIF-1 regulation under aerobic and

Figure 2.7. HIF-1α regulation under aerobic and hypoxic conditions. When oxygen is available proline 402 and proline 564 hydroxylate HIF-1 α. Proline hydroxylations enables VHL binding and ubiquitin-mediated degradation by the proteasome. Asparagine hydroxylation then prevents binding to p300/CBP and a derivative of HIF-3α called IPAS competes for HIF-1 α binding. HIF-1α binds to p300/CBP and SRC-1 and then translocates to the nucleus, activating genes possessing hypoxia responsive elements (HREs) such as EPO and VEGF (Giaccia et al., 2004).

Both cultured cells and isolated, perfused and ventilated lung has shown an exponential increase in HIF-1α levels at O_2 concentrations < 6%, which is not explained by the known biological function of hydroxylases. Most adult human tissues possess oxygen concentrations in the range of 3-5%, with any decreases occurring along the steep portion of

the dose-response curve, allowing for a graded response to hypoxia (Semenza et al., 2012). A recent gene expression analysis demonstrated that 200 genes were induced by HIF, with another 250 repressed by this transcription factor (Semenza, 2003). HIF targets include those related to vasomotor control (Nitric Oxide Synthase-2, NOS2), angiogenesis (VEGF, FLT1), blood and iron metabolism (EPO, transferrin, transferrin receptor), cell proliferation (IGF-1; IGFBP-1, transformin growth factor (TGF) and energy metabolism (Glucose transporters 1-4, lactate dehydrogenase-A, phosphoglucokinase-1, pyruvate kinase and many others (Radcliffe et al., 1998; Semenza et al., 1991; Semenza et al., 1994; Semenza et al., 1996; O'Rourke et al., 1997). It has been postulated that HIF-1 is exploited by a variety of physiological adaptive mechanisms requiring metabolic changes (Treinin et al., 2003), and that this transcription factor is important for not only adaptation to low oxygen environment, but also is integral in the adaptation to heat (Maloyan et al., 2005). The role of HIF-1 in heat acclimation is discussed later (Section 2.7.2).

2.3.2.3 Intermittent hypoxic training

The use of daily intermittent hypoxic exposure (IHE) has gained popularity in recent years, for both improving performance at sea-level via the live-high train low (LHTL) model (Millet et al., 2010), although the efficacy of this approach is still up for debate (Siebenmann et al., 2011; Duke et al., 2012), and to provide a degree of prior acclimation before travelling to altitude. Both hypobaric and normobaric hypoxia has been used to administer IHE. Hypobaric hypoxia is induced by ascent to the desired altitude, or via a hypobaric chamber simulating the conditions of a given altitude. Alternatively, normobaric hypoxia is achieved by addition of nitrogen into the inspired gas in order to reduce the PO_2 to the equivalent level of altitude (Muza, 2007). For logistical purposes normobaric hypoxia removes the need for continuous travel between altitudes and sea level (if a LHTL approach for enhancing sea

level performance is being sought). Hypoxic units are also relatively small and inexpensive compared to other methods.

The IHE protocol is defined by the severity of the simulated altitude, the duration of the exposure, and the total number of sessions (Millet et al., 2010). The effectiveness of IHE can be assessed by examining the physiological and haematological responses following an exposure (such as increased minute ventilation, SpO_2, Hb and Hct), or by functional outcomes such as improved endurance performance at altitude, or reductions in symptoms of acute mountain sickness. Typically, the outcome variables in such studies are assessed within the first 24-48 hours after the final IHE session. Thus the persistence of any conferred acclimation, and the time course of its decay, has yet to be fully investigated. There are also few studies that have used a normobaric IHE and then assessed its efficacy in improving tolerance to an ecologically valid hypobaric hypoxic exposure. The only controlled studies that reported improved outcomes for AMS and/or physiological performance used hypobaric procedures to improve later hypobaric exposure (Beidleman et al., 2003, 2004, 2008). A recent, well controlled study examined whether IHE delivered via 21 hours of normobaric hypoxia (PO_2 = 90mmHg for 2hr/day and PO_2 = 110mmHg/3020m asl, for 1 hr/day) over 7 consecutive days reported the attainment of ventilatory acclimation (Beidleman et al., 2009). However this did not transfer to hypobaric hypoxia, with the impaired time-trial performance still observed post IHE (Beidleman et al., 2009). Schomer et al., (2010) reported no differences in arterial blood gases or AMS symptoms after 14-18hours of normobaric hypoxia (12-16% O_2, 70-90mins/day, 3 days/week for 4 weeks, overnight stay at 3611m) when participants were relocated to a hypobaric environment compared to a control group (Schomer et al., 2010). This lack of improvement in the hypobaric environment was attributed to the longer than 24-hour return to sea level conditions prior to the hypobaric

testing, which was the common time period for hyperbaric testing in other studies (Beidleman et al., 2004, 2008). They suggest this may have led to a loss of any ventilatory acclimation (Schomer et al., 2010). However, ventilatory acclimation has been shown to persist for up to 1-month after the removal of normobaric hypoxic treatment when assessed in normobaric hypoxia (Katayama et al., 2005). Sleeping at ascending normobaric altitudes (2200 – 3100m) over 7 consecutive nights, providing an IHE time of 52.5 hours, also has been shown to induce little benefit upon later hypobaric hypoxic exposure (4300m). This IHE duration is twice as long as the minimal total hypobaric treatment required to induce beneficial effects to hypobaric hypoxia (Beidleman et al., 2009), and 3 times as long as other studies investigating normobaric to hypobaric acclimation (Fulco et al., 2011). Sleeping in normobaric hypoxia produced a measurable ventilatory acclimation to normobaric conditions. However this did not appear to be present during waking hours in the hyperbaric environment. There was also no difference between the experimental and sham groups for exercise performance in hypobaric hypoxia (Fulco et al., 2011). Reductions in AMS symptoms occurred as a result of time spent in the hypobaric environment, with the experimental group no different from sham.

It would seem that, although repeated exposure to normobaric hypoxia improves tolerance to later normobaric exposure, it provides limited acclimatory benefits for exposure to hypobaric hypoxia. This is a surprising finding given the increase in commercial activity regarding the use of normobaric hypoxic exposures preparing individuals for high-altitude treks. See for example the altitude centre in London (https://www.altitudecentre.com). Normobaric and hypobaric hypoxia therefore cannot be treated as interchangeable, and information regarding normobaric studies may not translate to real-world scenarios (Fulco et al., 2011). The reasons for the divergent responses between normobaric and hypobaric hypoxia are not presently

known (Beidleman et al., 2014). It is possible that nitric oxide (NO) metabolism plays a role, as exhaled NO has been shown to be lower in hypobaric conditions compared to normobaria (Hemmingson and Linnarsson, 2009). The lower NO concentrations in expirate were later shown to be mediated by barometric pressure (Kerckx et al., 2010). A reduction in exhaled NO could be induced by a higher back-diffusion to the alveoli and then to the haemoglobin in hypobaria, suggesting that more NO is likely to be captured to the blood compartment in hypobaric conditions (Millet, 2012). The differences in NO may be related to the reduced ventilation seen in hypobaria. Lower tidal volumes and higher respiratory frequencies alongside reduced PET_{O2} and PET_{CO2} have been observed in hypobaric conditions (Savourey et al., 2003; Loeppky et al., 2005). Nitric Oxide Synthase (NOS) has an excitatory influence on respiration during hypoxia, and when it is inhibited the ventilator responses to hypoxia are reduced (Gozal et al., 1996). Therefore the lower minute ventilation observed in hypobaric conditions by be an indirect consequence of the reduced NO bioavailability, itself caused by the reduction in barometric pressure.

2.3.3 Heat acclimation and hypoxic acclimation - common and divergent responses

In summary, the process of heat acclimation is a transition between two distinct phases: an early inefficient phase, to a later more efficient acclimatory homeostasis (Horowitz et al., 2002; Horowitz et al., 2003). In the early phase, short-term heat acclimation (STHA), an increase in excitability of the autonomic nervous system compensates for impaired cellular performance. Once long-term heat acclimation (LTHA) has been reached, an enhanced cellular function decreases the excitability of the ANS and increases the dynamic thermoregulatory range, which is characterised by decreased heat production and the reductions in temperature sensor thresholds heat dissipation mechanisms to be induced

These adaptations eventually manifest as reductions in physiological strain during submaximal exercise, as evidenced by a reduced heart rate and T_{core}. The reductions in exercising HR are thought to be in part, mediated by plasma volume expansion (Garrett et al., 2009). Heat acclimation has been shown to improve time trial performance in both heat and normothermic conditions via increased efficiency (Lorenzo et al, 2010).

During adaptation to hypoxia, the early responses include increased ventilation, leading to a respiratory alkalosis and leftward shift in the oxygen-dissociation curve. Over time, hypoxia induces polycythemia secondary to EPO release at the kidney to increase arterial oxygen content (Richalet et al, 1994). Adaptation to hypoxia does not however, restore or improve $\dot{V}O_{2\ max}$ despite marked increases in O_2 carrying capacity (Bender et al., 1988; Calbet et al., 2003). The lack of an acclimation effect on $\dot{V}O_{2\ max}$ has been attributed to a decrease in maximal cardiac output offsetting an increase in arterial content (Favret et al., 2001). However, during submaximal exercise in hypoxia HR can be reduced following acclimation.

The importance of the opposite alterations in plasma volume (increasing following heat, and decreasing following hypoxia) on establishing whether cross-acclimation between heat and hypoxia occurs in whole body humans have yet to be addressed. It has been suggested that the increased physiological efficiency accrued after heat acclimation, coupled with shared cellular adaptive mechanisms between heat and hypoxia may override this seemingly contradictory adaptation to blood volume (Heled et al., 2012), at least in the short term.

2.4 Heat shock proteins and the heat shock response

Heat shock proteins (HSPs) are a group of highly conserved proteins, which are named according to their molecular weight. Heat shock proteins play an important role in a multitude of cellular processes and adaptive cascades and appear to play a major role in

cytoprotection by regulating apoptosis (Sreedar and Csermely, 2004). There has been a recent increase in interest in the study of these proteins, in particular in response to exercise performance (Fehrenbach et al., 2005), and their role in both heat (Yamade et al., 2007; McClung et al., 2008; Kuennen et al., 2010) and altitude acclimation strategies (Taylor et al., 2012, 2013). A review of each member of the heat shock protein family is beyond the scope of this review, so the reader is directed to many excellent recent reviews on the subject (Kiang and Tsokos, 1998; Kregel, 2002; Morton et al., 2008; Yamada, 2008; Nobel et al., 2010).

2.4.1 Heat shock protein 72 (HSP72)

The HSP70 family of proteins has four major members with HSP75 (mitochondrial HSP70) and HSP78 (Glucose regulating protein 78, Grp78, BiP) performing chaperone functions in the mitochondria and endoplasmic reticula respectively. The remaining family members, are the constitutive heat shock cognate 70 (HSC70 or HSP73) and the highly inducible isoform HSP70 (HSP72). Specifically, the highly conserved and inducible HSP72 performs multiple functions related to protein homeostasis (Goldberg, 2003; Arslan et al., 2006). Protein specific functions include *de novo* folding (Fink, 1999), refolding (Hartl, 1996) and degradation (Garrido et al., 2001), in addition to extracellular pro-inflammatory (Pockley, 2003; Hickman-Miller and Hildebrand, 2004; Asea, 2005) and intracellular anti-inflammatory responses (Ianaro et al., 2001). These specific functions enable HSP72 to provide acquired thermotolerance (Sandstrom et al., 2008), cytoprotection (Garramone et al., 1994; Lepore et al., 2000; Suzuki et al., 2000; Maglara et al., 2003; McArdle et al., 2004) and cross tolerance between different stressors that invoke HSP72 as part of the stress response (Levi et al., 1993; Horowitz, 2002; Arieli et al., 2003; Shein et al., 2005; Shein et al., 2007)

Elevated levels of HSP72 have been postulated to provide tolerance to the biochemical stresses that accompany exercise (Locke and Noble, 1995; Morton et al., 2009c).

2.4.2 Regulation and expression of HSP72

HSP72 has been induced by a range of environmental, pathophysiological and non-stress conditions, including hyperthermia (Ritossa et al., 1962), hypoxia (Patel et al., 1995; Weinstein et al., 2004), oxidative stress (Kukreja et al., 1994), exercise (Locke and Nobel, 1995) and hyperbaria (Yogaratnam et al,. 2007). Substrate depletion (Febbraio and Koukoulous, 2000), immunological disorders (Hickman-Miller and Hilderbrand, 2004) and acidosis (Gapen and Mosely, 1995; Peart et al., 2012) have also been shown to induce HSP72 expression. Figure 2.8 displays these HSP72 inducers.

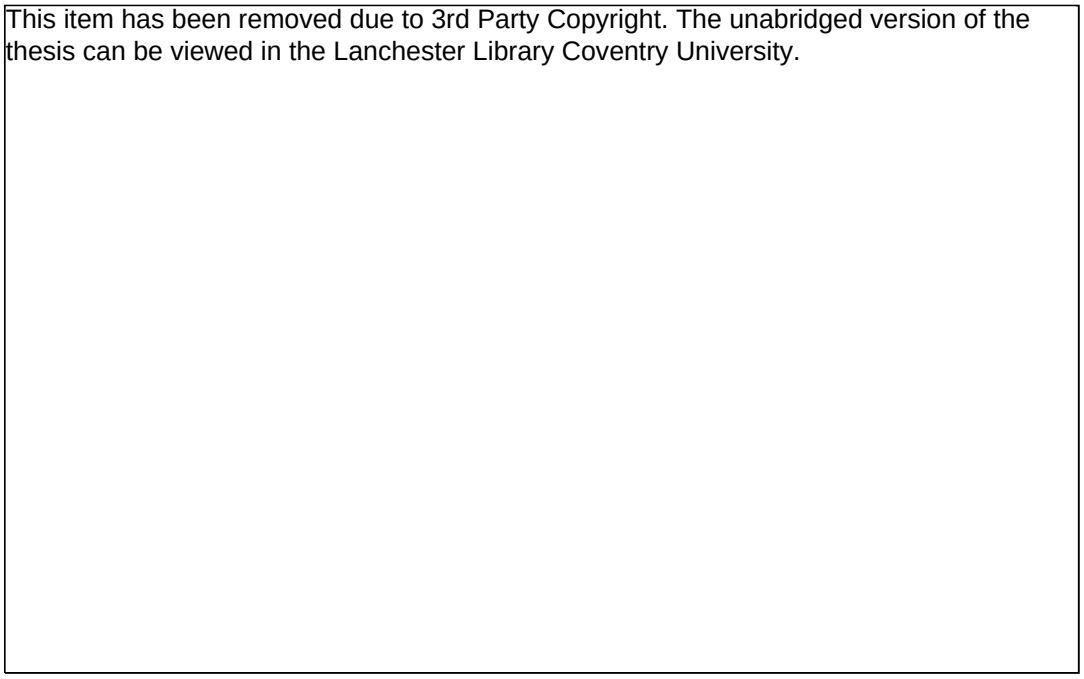

Figure 2.8. Conditions that induce the heat shock response. Heat shock gene expression represented here by the activation of heat shock factor and resulting binding to the heat shock element, with successful binding inducing HSP72 expression. Taken from Morimoto (1998).

There are four components in the mechanism of HSP72 expression: The first potent stimulus for HSP72 expression requires an accumulation of denatured proteins within the intracellular environment (Palleros et al., 1991); secondly, the presence and physiological requirement of other HSPs (co-chaperones); thirdly, the involvement of ATP hydrolysis and/or binding initiated activation (Wang et al., 1993; Szabo et al., 1994); and finally, differential regulation of target peptides and modulation of that information or assembly (Gabai and Sherman, 2002). Thus the transcription, translation, translocation and synthesis of human HSP72 are complex and multifaceted, requiring many intracellular and extracellular collaborations (Asea, 2007).

The mechanism of HSP72 gene expression is regulated by heat shock transcription factor 1 (HSF1). In unstressed conditions, HSPs bind to monomeric HSF1, which in this form is in a suppressed state. Establishment of this state is important for the maintenance of both protein homeostasis and coping with cellular insult (Katschinski et al., 2004). Under non-stressed conditions, HSP72 is bound to HSF1 in the cytosol. However, during stress insult HSPs dislocate from HSF1 and bind instead to misfolded proteins. This suggests that HSPs have a greater affinity for misfolded proteins than HSF1. Once separated from HSPs, HSF1 is phosphorylated, modulating its conformational structure, allowing it to translocate across the nuclear membrane and enter the nucleus, binding to HSP genes (Cotto and Morimoto, 1999). In this state HSF1 has a high binding affinity for DNA sequence elements, called heat shock elements (HSEs) in the promoter region of the HSP gene. Once the stress abates, the trimeric forms of HSF1 dislocate from the HSE and are returned back to HSF1 monomers with the inability to bind to DNA. An increase in HSPs as a result of this process are also thought to be negative regulators of HSF1, binding to HSF and preventing further trimerisation once sufficient HSPs have been synthesized to deal with the stress (Wu et al., 1993).

2.4.3 Function of HSP72

The primary function of HSPs is to assist in the correct folding and refolding, and protection of partially denatured proteins by shielding hydrophobic regions, thereby preventing protein aggregation and promoting proper folding. HSPs are constitutively expressed and carry out these functions as part of the normal life cycle of the cell. Mammalian HSPs have been shown to bind to a large number of newly synthesized polypeptides, thought to comprise 15-20% of total protein (Kregal, 2002). The homeostatic regulation of *de-novo* protein synthesis is managed by HSP73, whereas under conditions of stress the response is overseen and orchestrated by HSP72. Overexpression of HSP72 has been reported for both heat (Powers et al., 2001) and hypoxia (Benjamin et al., 1990; Wang et al., 2006). The predominant function of HSP72 is to act as a molecular chaperone (Arrigo, 2005; Voelly and Boellmann, 2007). Chaperones bind to denatured or unstable proteins in order to stabilize them (Gabai and Sherman, 2001). Chaperones have also been shown to bind to nascent polypeptides soon after they exit the ribosome, masking the hydrophobic residues from any unwanted interactions (Young et al., 2004; Bakau et al, 2006). The chaperone function is not only restricted to denatured proteins. HSPs are also involved in the functional maturation of steroid receptors, helping the process of folding, maturation, nuclear trafficking and assembly of transcriptional processes (Kiang and Tsokos, 1998). HSPs mediate cellular trafficking of proteins into different compartments of the cell and mediate endocytosis (Asea, 2003). They play a yet to be defined role in exocytosis and regulate protein degradation by interfering with pathways associated with this function, such as the lysomomal and ubiquitin-proteome systems (UPS) (Salvador et al., 2002; Majeski and Dice, 2004). HSPs also promote the processing of G-protein coupled receptors (GPCR) to the plasma membrane. This is relevant to human health as these receptors are the targets of 30% of marketed drugs (Chapple, 2003). HSPs therefore

possess a multitude of functions within the cell, which are vital for maintaining cellular homeostasis.

2.4.4 Extracellular HSP72

HSP72 has a functional role when released by a variety of cells (Febbraio, et al., 2002; Broquet, et al., 2003; Hunter-Lavin, et al., 2004; Lancaster, 2004) into the circulation (eHSP72) (Pockley, 1998). eHSP72 has been suggested to have an immunological function (Moseley, 1998; Asea, 2000; Bethke, et al., 2002; Campisi and Fleshner, 2003; Fleshner, 2003). Pockley et al., (2000) first reported HSP72 was detectable in the circulation of humans. People suffering from renal disease, hypertension, and atherosclerosis have chronically elevated basal levels of eHSP72 relative to age matched, healthy controls. In addition to this, Dybadahl et al., (2001) found that patients with coronary artery disease have an acute increase in eHSP72 in response to coronary bypass surgery. Later, several studies reported that organisms absent of disease also increased the concentrations of eHSP72 in blood after exposure to acute physical and psychological stressors (Fleshner et al., 2003; Campisi et al., 2003a 2003b, 2003c). eHSP72 has been proposed to stimulate innate immunity (Matzinger, 1994) and act as a danger signal resulting in increased immune responses and facilitation of host pathogenic challenges (Fleshner & Johnson, 2005). eHSP72 has been reported to attach to the surface of monocytes stimulating the production of several cytokines (Asea, 2000). The changes in eHSP72 concentrations are thought to have a subsequent effect on the cytokine cascade and therefore the inflammatory response (Marshall, et al., 2006). eHSP72 has little impact on proinflammatory cytokines if there is no pathogenic challenge. Following pathogenic challenges eHSP72 may help eliminate bacterial pathogens (Fleshner & Johnson, 2005). Circulating eHSP72 expression may play a role in protecting the intestinal epithelial tight junction barrier in caco-2 cells when exposed to moderate increases

in temperature (37-41°C). Preserving tight junctions prevents large amounts of endotoxin release into the portal vein, activation of macrophages and the subsequent cytokine cascade (Lambert, 2004; Lim and Mackinnon, 2006).

2.4.5 Mechanisms of eHSP72 release

While the molecular mechanisms of HSP synthesis are well understood, less is known regarding how HSPs are released into the extracellular milieu. HSPs do not possess the leader sequence normally involved in secretion, with their release likely involving non-classical mechanisms (Ogawa and Fehrenbach, 2010). Early theories on the release on eHSP72 were centred on necrotic/lycic cell death (Basu et al., 2000). Although studies have shown necrotic cell death to lead to eHSP72 release (Basu et al., 2000; Sauter et al., 2000; Berwin et al., 2000) HSP72 has been shown to be released in the absence of necrosis. Glial cells (Guzhova et al., 2001), B cells (Clayton et al., 2005), tumor cells (Gastpar et al., 2005), and human peripheral blood mononuclear cells (PBMC) (Lancaster and Febbraio, 2005) all exocytotically release eHSP72 in the absence of cell death. Lancaster and Febbraio (2005) demonstrated that exosomes gradually increase in both culture mediums (RPMI 1640, 0% fetal bovine serum) and PBMC cell culture in a time dependant manner, while HSP72 content of exosomes also increased (Lancaster and Febbraio, 2005). Furthermore, many studies have shown an elevated circulating level of eHSP72 within 10-25 minutes of stressor onset, a speed which suggests that the protein induction/necrosis release pathway is unlikely (Johnson and Fleshner, 2006). It is believed that an intracellular HSP72 is the potential source of eHSP72 release, as the release may not depend on the translation of new intracellular protein (Fleshner et al., 2003). However HSP72 content in the cells does not always reflect the magnitude of eHSP72 (Lancaster and Febbraio, 2005).

Evidence suggests that hormone receptor-mediated exocytotic pathways may exist, increasing eHSP72 in times of stress. Previous studies have indicated that a close relationship exists between neuroendocrine activation during stress and release of eHSP72. Johnson, et al., (2005) produced data supporting the involvement of catecholamines in the eHSP72 response to tail shock in rats through the use of α and β adregenic blockades in a rat model. Rats pre-treated with a non-selective adregenic receptor (ADR) antagonist (labetalol) or a selective α1-ADR antagonist (prazosin), but not a selective β-ADR antagonist (propanol) prior to tailshock, blocked the rise in eHSP72 (Fleshner and Johnson, 2005). The authors also demonstrated that administration of a selective α1-ADR agonist (phenylephrine) to non-stressed rats elicited elevated eHSP72, whereas a β-ADR agonist had no effect on eHSP72 release. Therefore, as nor-adrenaline binds with a higher affinity than adrenaline (Hardmen et al., 2001), and an adrenalectomy, which depleted 95-99% of adrenaline (Hessman et al., 1976; Vollmer et al., 1995), has been shown to have no effect on stress induced eHSP72 expression (Johnson et al., 2005), it can be concluded that the increase in circulating eHSP72 in response to stress is, at least in part, due to a sympathetic nervous system activation and the release of noradrenaline. This acts at α1-ADR to increase the concentration of circulating eHSP72 (Figure 2.9).

Whitham, et al., (2006) has also provided support for the involvement of catechlolamines in the stimulated release of eHSP72 in a human model. Because the rate of exocytosis of these vesicles are calcium dependant (Hightower & Guidom, 1989) and α-ADR are thought to mediate their actions thought alterations of intracellular calcium (Garcia-Sainz et al., 1999), adregenic stimulation of exosomal release may be a mechanism by which cells release HSP72 (Whitham et al., 2007). Mambula and co-workers (2006, 2007) report the endolysosome pathway as another alternative release mechanism. HSP72 release involves the

transit through the endolysosomal compartment and requires ABC family transporter proteins, followed by the stress induced HSP72 release via extracellular ATP (eATP). That downhill running increases eHSP72 (Peake et al., 2005) whereas eccentric elbow flexor exercise does not (Hirose et al., 2004) indicates factors other than necrosis and damage lead to eHSP72 release. The conflict in these results may be explained by the endolysomal pathway; in this mechanism eATP regulates HSP72 release from ABC family transporters and thus muscle damage does not contribute to increased eHSP72. Instead eHSP72 increases with eATP (Ogawa and Fehrenbach, 2010).

2.4.6 Sources of extracellular HSP72

Potential sources for the release of HSP72 into the circulation include skeletal muscle, brain, heart, leukocytes and the hepatosplanchnic tissue (Mosely, 2000; Walsh et al., 2001; Febbraio et al., 2002; Hunter-Lavin et al., 2004; Lancaster et al., 2004; Ferenbach et al., 2005). The concentration of HSP is upregulated in these aforementioned cell types following exercise, although the increase in eHSP72 in serum or plasma precedes any increase in HSP72 mRNA and protein in muscle tissue (Walsh et al., 2001). eHSP72 is found in arterial, but not venous, blood flow in a blood flow in a contracting leg model (Febbraio et al., 2002) indicating that human skeletal muscle does not appear to secrete HSP72. The hepatosplanchnic tissue substantially contributes to circulating eHSP72 concentrations (Febbraio et al., 2002), and significant exercise induced increases from the liver have been observed in animals (Salo et al., 1991; Kregel and Mosely., 1996).

Figure 2.9 Proposed mechanisms of eHSP72 release. (1) Tissue damage produces the necrotic release of cytolic HSP72 into the extracellular space. (2) activation of the sympathetic nervous system during stressor exposure results in the release of nor-adrenaline and activation of α1-ADR. Stimulation of α1-ADR results in an increase in intracellular calcium, which may stimulate the release of exosomes containing HSP72. (3) Secretion through endolysosomes and release when the endolysosomes fuse with the cell surface. This type of HSP72 secretion requires HSP72 entry into endolysosomes via ABC-family transporters, where they localise with intravesicular Cathespine D. eATP coordinates the lysosomal pathway through purinegic receptors (P2X), and the organelles are then transported to the cell surface. Fusion of HSP72 containing endolysosomes with the cell surface results in the localisation of LAMP1 on the plasma membrane and release of HSP72 with other proteins such as cathspin D (Mambula and Calderwood, 2006; Ogawa and Fehrenbach, 2010).

Leukocytes are also able to actively secrete HSP72 (Hunter-Lavin et al., 2004), and their intracellulare expression is increased by exercise (Fehrenbach et al., 2000). Extracellular ATP has been shown to activate P2X receptors, which allow cations and other small molecules to pass through the transmembrane (Di Giroamo et al., 2005). P2X7 receptors are possessed in many human cells, including macrophages and lymphocytes (Gu et al., 2000), whereas P2X6 receptors are present in skeletal muscle (North et al., 2002). Thus P2X7 may be an important feature in HSP secreting cells. The P2X receptor in the liver has an unusually high sensitivity to ATP (North et al., 2002), thus P2X receptors in the endolysosomal pathway are a likely source of HSP72 release.

2.5 The HSP72 response to exercise and environmental stress

Early research into exercise and HSP72 focused on cell and tissue isolates/cultures which were manipulated to mimic individual and combined components of the biological consequences of exercise. For example, acidosis, hyperthermia, glucose deprivation and oxidative stress are all exercise-associated permutations of homeostasis and have each been examined in relation to their individual and combined roles in HSP72 expression (Hammond et al., 1982; Locke et al., 1990; Salo et al., 1991). Research then moved into whole body animal models, and latterly, human *in vivo* studies. This section of the literature review will first consider the early pioneering animal exercise models before addressing the *in vivo* human studies pertaining to peripheral blood mononuclear cells (PBMC) and extracellular HSP72, as these are to be the tissues examined in later experimental work within this thesis.

2.5.1 Animal HSP72 responses to exercise

Hammond et al., (1982) provided the first examination of *in vivo* exercise related inductions of HSP72. Rats were swam to exhaustion in normothermic (59mins), hyperthermic (41mins,

42°C), hypothermic (87mins, 20°C) conditions. A further group of animals had their descending aorta banded to create an ischemic environment at the myocardium (Hammond et al., 1982). The control and hypothermic animals demonstrated no synthesis of HSP72 protein, whereas the hyperthermic and banded animals had a clear increase in HSP72 protein after the stress exposure (Hammond et al., 1982). Later, Salo et al., (1991) observed elevated HSP72 mRNA in the skeletal and cardiac muscle of rats post exercise, with maximal elevations occurring between 30 and 60 minutes post exercise in skeletal muscle, and 6 hours post exercise in cardiac tissue (Salo et al., 1991). This increase in HSP72 minus an exogenous temperature stimulus was thought to be a physiological response to the exercise induced endogenous temperature increases and increases in oxidative stress (Salo et al., 1991). However the effect of exercise on total protein was not assessed. Treadmill running induced time-dependent higher HSP72 protein expression during and after exercise in muscle tissue, PBMCs, and spleen cells when compared to non-exercise control animals (Locke et al., 1990; Locke et al., 1991).

Whilst these early studies contributed significantly to the understanding of the *in vivo* HSP response to exercise, the effect of exercise without the associated increases in metabolic heat production and elevations in body core and muscle temperatures were not addressed. Normothermic treadmill running in rats was shown to induce elevations in HSP72 within locomotor muscle (soleus and gastrocnemius) whereas non-locomotor muscles demonstrated no increases in comparison to control tissues, further demonstrating that exercise without an external heat load can induce HSP72 expression (Skidmore et al., 1995). To address the role of exercise with and without thermal stress another group of animals completed an identical work bout whilst under hyperthermic conditions. When compared to exercise or hyperthermia alone, the combination of the two elicited a 200% greater increase in HSP72 protein

(Skidmore et al., 1995). The magnitude of the HSP response to exercise was shown to be both duration and intensity dependent (Demirer et al., 1999), and the increases in post exercise HSP72 were inversely related to basal values, a finding that had been consistently reported both *in vitro* (Vince et al., 2010) and *in-vivo* (Gjovaag and Dahl, 2006).

2.5.2 Human HSP72 response to exercise

Blood, and therefore peripheral blood mononuclear cells are exposed to all body compartments, and by extension all alterations in body temperature and oxygen metabolism. This tissue is a useful and informative cell type when addressing gene, mRNA and total protein expression in humans as it can easily be obtained from human volunteers, yielding sufficient cells for analysis from 100μL of venous blood. The recent increase in the use of flow cytometry in the assessment of exercise-induced changes in HSP72 concentrations is largely due to the highly specific identification of HSP72 afforded by the technique. This method is also quick, with analysis of live cells achievable within 90 minutes of sample collection, and both sensitive and reliable (Bachelet et al., 1998). Typically, early research utilizing PBMC samples used western blot to assess leukocyte HSP72 expression, with researchers finding no change in leukocyte HSP72 expression following treadmill running (Ryan et al., 1991; Shastry et al., 1992) or cycle ergometer exercise (Chang et al., 1998). This method lacks the specificity and sensitivity of flow cytometry, which allows separate analysis of granulocytes, lymphocytes and monocytes, which have each been shown to display different stress responsiveness (Bachelet et al., 1998; Nolan and Sklar et al., 1998; Vignali et al., 2000; Fehrenbach et al., 2005; Herzenberg et al., 2006; Ireland et al., 2007).

Each PBMC subgroup has been shown to possess different stress responsiveness following a bout of exercise. It has been consistently demonstrated that lymphocyte HSP72 expression is

unaffected post exercise (Fehrenbach et al., 2000; Fehrenback et al., 2000; Fehrenbach et al., 2001; Whitham et al., 2004; Peart et al., 2011; Peart et al., 2013), thus modern literature has typically focused upon the more stress responsive monocyte and granulocyte cells. Post half marathon (90.34 mins) both granulocyte and monocytes showed an increase in HSP72 (Fehrenbach et al., 2000; Fehrenbach et al., 2000). It was also noticed that trained volunteers had lower basal expression of both monocyte and granulocyte HSP72 compared with less trained volunteers. Further to this, an *ex-vivo* heat shock of basal PBMC samples revealed that the trained PBMC sample was more responsive to the stressor, indicated by a significantly accelerated HSP72 mRNA synthesis in the trained group (Fehrenbach et al., 2000). Thus, despite the lower basal HSP72 expression, trained individuals are better equipped to oppose a non-lethal thermal stressor via an accelerated HSP72 transcription. This was also shown to be of a greater magnitude compared to the lesser-trained samples. It is possible that extensive aerobic training down-regulates both *m*HSP72 and *g*HSP72 expression, which when under a homeostatic challenge, is more rapidly accessible to improve cellular protection.

Recently, the effects of short duration, supramaximal exercise on HSP72 have been examined (Peart et al., 2011). A 4-minute bout of cycling, in which participants were instructed to provide a maximum effort, induced a 50% increase in mHSP72 30 minutes post exercise, and this effect was still evident 2 hours later. This work bout induced significant metabolic stress, with post exercise blood lactate values in excess of 17mmol$\bullet$L^{-1}, and blood pH reduced to below 7.2 (Peart et al., 2011). These authors found that a pre-exercise induced alkalosis, achieved via sodium bicarbonate ingestion, significantly attenuated the post exercise mHSP72 response. This is the first *in vivo* evidence in a human model to directly address the role acidosis has on stimulating HSP72 expression, an inducer of the HSR previously

examined *in vitro* (Gapen and Mosely, 1995; Narasimhan et al., 1996). A more prolonged, 90-minute intermittent cycling exercise bout was shown to induce mHSP72 post exercise (45% increase), but on this occasion the prior ingestion of sodium bicarbonate had no effect on post exercise mHSP72. It is likely that this more prolonged work bout perturbed other homeostatic mechanisms that are known to be potent inducers of HSP72, such as increased T_{core} and muscle temperature. These features would be much reduced in a 4-minute exercise bout compared to 90-minutes (Peart et al., 2013). It is interesting to note that the post exercise increases in mHSP72 was comparable between two very different exercise bouts (4 minutes all out compared to 90 minutes of alternating workload). This prolonged work bout lead to much lower peak blood lactate values and alterations in pH, possibly accounting for the more pronounced post exercise changes after 4 minutes of exercise (Peart et al., 2013).

In summary, PBMCs represent an easily accessible, stress responsive tissue for investigations into HSP72 and exercise. It is now accepted that flow cytometry is a fast, reliable and more sensitive measure of HSP72 when compared to commonly used western blot or RT-PCR analyses. Studies employing flow-cytometry based analysis have shown that exercise leads to increases in monocyte and granulocyte increases in HSP72, with lymphocyte expression unaffected. Training status appears to reduce basal HSP72 expression. However the response to exercise stress is more efficient than seen in lesser-trained individuals, with rapid mRNA expression and a greater magnitude of protein expression observed in trained groups.

2.5.2.1 Human extracellular HSP72 and exercise

The effects of exercise on extracellular HSP72 has been extensively reported in the literature using both serum and plasma derived samples. This despite the tissue of release, nor its physiological function being known with certainty, making inferences about the relevance of

exercise induced eHSP72 appearance problematic. The collection of serum or plasma for later analysis via ELISA represents an easy collection method, with little technical expertise and potentially costly optimisation required for later analysis compared to the separation and analysis of PBMCs. This may partly explain the greater abundance of literature examining eHSP72. There is a considerable variability in both the resting basal values of eHSP72 and the magnitude of the exercise response. Both "in house" and commercially bought kits have been used, introducing a large inter and intra assay variability. A recent improvement in kit sensitivity from 0.8mg•mL^{-1} to 0.2ng•mL^{-1} further adds to the issue of data variability.

The type of specimen used also affects eHSP72 results with serum yielding lower basal values than those obtained via plasma (Whitham and Fortes, 2008). This means data, when represented as a percentage change, is often higher in serum-based studies. For example, treadmill based running protocols using a similar workload (70 and 75% $\dot{V}O_{2\ max}$) and exercise duration (60 minutes) have shown post exercise increases in serum eHSP72 of 685% (Walsh et al., 2001) and plasma eHSP72 of 170% (Fehrenbach et al., 2005), clearly demonstrating the effects of sample choice on eHSP72 responses. It has also been demonstrated that different blood coagulation tubes yield different plasma eHSP72 values, with EDTA derived plasma yielding a 137% higher values than heparin counterparts (Whitham and Fortes, 2008). Despite these obvious problems with sample collection and subsequent analysis, there are some clear trends within the literature.

Running on a treadmill in the laboratory, on the track, or as part of a competition induces intensity and duration dependent increases in eHSP72. Different running loads have been examined, with a competitive half marathon inducing a plasma eHSP72 increase of 725%, versus 175% when running for 60 minutes at 75% $\dot{V}O_{2\ max}$, or 140% when running for 120

minutes at 60% $\dot{V}O_{2\ max}$ (Fehrenbach et al., 2005). More extreme bouts of exercise, such as an ironman competition (2100%; Suzuki et al., 2006) or a 100km ultra marathon with completion time around 540 mins (1600%; Gomez-Merino et al., 2006) also indicate the important role of intensity and duration in post exercise eHSP72 expression. Downhill running has been shown to induce a 50% greater peak increase in eHSP72 compared to neutral grade running, which was correlated to biochemical markers of muscle damage (Peake et al., 2005). The authors fail to provide a mechanistic explanation for this increase, and the origin of exercise induced eHSP72, and therefore the relevance of such increases have not been sufficiently addressed to allow meaningful inferences to be made.

The sum exercise stress of cycling is less than that of running due to its non-damaging nature. Thus cycling induced elevations in eHSP72 are much lower than those reported for running protocols. Projects have demonstrated a zero (Febbraio et al., 2002) or 300% (Fischer et al., 2006) increases in serum eHSP72. Low increases similar to the detection limit of the assay available at the time have been reported post cycle ergometer exercise (Febbraio et al., 2002; Febbraio et al., 2004; Lancaster et al., 2004). For example, exercise induced changes of 0.88ng•mL^{-1} (Febbraio et al., 2002) and 0.9ng•mL^{-1} and 1ng•mL$_{-1}$ are only 0.1ng•mL^{-1} above the limit of detection for the assay and would explain why no basal eHSP72 was detected in participants in these studies.

2.5.3 Human HSP72 response to heat stress

There a surprisingly few studies investigating the PBMC HSP72 responses to acute heat and exercise stress in humans, though there is a clear and repeatable trend for hyperthermic exercise inducing a significantly greater HSP72 expression compared to exercise alone. A bout of hyperthermic running (60 minutes at 90% anaerobic threshold, 28°C) induced a 30%

increase from rest in mHSP72, values also reported for the thermoneutral control group (Fehrenbach et al., 2003). Interestingly, the control group (same exercise bout at 18°C) displayed different HSP72 protein expression kinetics, despite an almost identical 40% increase in post exercise HSP72 mRNA (Fehrenbach et al., 2003). 24 hours after the run, mHSP72 was 40% higher than baseline values in the hyperthermic group, whereas mHSP72 had returned to near basal levels (10%) in the control group. Post exercise elevations were maintained for the hyperthermic running group at the final measurement 48 hours post run (20%), and had returned to basal values in the control group (Fehrenbach et al., 2003). It is possible that the added stress of hyperthermia to exercise induced a greater transcription rate of HSP72 protein in anticipation of sustained cellular insult, leading to its overexpression in the time period directly after removal from a stressor.

More recently, in a well-controlled study utilizing increments in T_{core} as sampling points, the effects of training status on the mHSP72 response to uncompensable heat stress was examined (Selkirk et al., 2009). The trained volunteers ($\dot{V}O_{2\ max}$ = 70 ± 2mL•kg^{-1}•min^{-1}) demonstrated a large increase in mHSP72 (100%) while walking with a T_c >38.5°C. This response was absent in the untrained group ($\dot{V}O_{2\ max}$ = 50 ± 1mL•kg•min^{-1}), findings which lend support to the earlier observations that trained individuals exhibit a more rapid and larger HSP72 response to stress (Fehrenbach et al., 2001).

Longer, fixed workload exercise bouts have also shown significant increases in PBMC derived HSP72. A 90-minute exercise bout treadmill running at 50% $\dot{V}O_{2\ max}$ (40°C, 45%RH) produced 300% increases in HSP72 protein post exercise, and 350% 1hour post exercise (Magalhaes et al., 2010). Peak T_{core} during this work bout was 39.0°C, with a significant portion of the 90 minute work bout occurring with a T_{core} >38.5°C (42mins). Short

duration heat stress tests have also induced increases in HSP72, though these increases were not of the same magnitude as these earlier studies (Kuennen et al., 2011). This may be explained by the method of analysis (western blot) used to quantify HSP72 protein. Despite this, a 45-minute bout of walking exercise at 50% $\dot{V}O_{2\ max}$ induced a non-significant increase in HSP72 20%. During this study, the peak T_{core} obtained was <38.5°C, thus both the intensity of exercise (walking at 50% $\dot{V}O_{2\ max}$) and exogenous thermal strain (< 38.5°C) may not have been sufficient physiological strain to induce a HSR (Kuennen et al, 2011). A bout of treadmill running (60 minutes at 60% $\dot{V}O_{2\ max}$) in high ambient temperature (42°C, 20%RH) induced only a moderate increase in post exercise PBMC HSP72 (10%), increasing to 40 and 45% from rest at 1 and 4hours post exercise respectively (Gillum et al., 2013). The small response in relation to other studies may be explained by the sample analysis used. From examining the gating strategy of the flow cytometry analysis it appears the authors grouped all PBMCs together in the analysis. Therefore relative unresponsiveness to stress of certain cell subtypes may have diluted the observed HSP72 response. A retrospective analysis of the FACS files, separately gating monocytes, lymphocytes and granulocytes would not be possible in this instance. Unfortunately, none of these studies utilized a thermoneutral control condition to separate the effects of heat and exercise on HSP72 induction as these comparisons were not central to the study specific research questions (Magalhaes et al., 2010; Kuennen et al., 2011; Gillum et al., 2013).

2.5.3.1 Human extracellular HSP72 response to heat stress

Heat is an important mediator of eHSP72 induction. For example runners completing a 14km road race that finished with symptoms of exertional heat illness displayed a far higher increase (2900%) in eHSP72 compared to control participants that finished minus these symptoms (850%) (Ruell et al., 2006). The importance of exercise with and without an

elevated T_{core} for eHSP72 increases was carefully investigated by using a thermal clamping protocol during underwater running (Whitham et al., 2006). Participants completed 120 minutes of underwater running with T_{core} clamped (36.25°C) or unclamped (38.5°C), producing post exercise increases in eHSP72 of 130% and 215% respectively (Whitham et al., 2007). The finding of a significant increase in eHSP72 following thermally clamped exercise supports the notion that exercise factors other than heat can stimulate the releases of eHSP72. Aerobically trained individuals appear better equipped to deal with hyperthermic challenges. Whereas trained individual display elevated basal plasma HSP72 and a more rapid response to the increasing T_{core} associated with uncompensable heat stress during treadmill walking compared with untrained individuals (Selkirk et al., 2009).

Recently, the effects of matched exercise intensity and duration in 3 different levels of thermal stress on plasma eHSP72 responses was addressed (Gibson et al., 2013). Moderate intensity cycling exercise (90 minutes, 50% $\dot{V}O_{2\ max}$) was performed in thermoneutral (20°C, 50% RH), hot (30°C, 50% RH) and a very hot conditions (40°C, 40%RH). The protocol produced 2 distinct levels of overall physiological strain, which was reflected in the eHSP72 responses, with the very hot condition inducing a significantly higher eHSP72 response (172%) compared to thermoneutral (-2%) and hot (26%) conditions. The data indicate that both the rate and delta change in Tc were important factors in augmenting eHSP72 expression. Mechanistically, temperatures above 38.5°C at the hepatosplancnic viscera are the most important for increased eHSP72 concentrations, with eHSP72 release particularly dependent on the magnitude and duration above this threshold (Selkirk et al., 2008; Selkirk et al., 2009). This was also reported by Periard et al., (2012), whereby an exercise bout at 75% $\dot{V}O_{2\ max}$ showed a relationship between eHSP72 and increased rate of T_{core} rise. The authors suggested the greater metabolic demand produced this effect, with similar eHSP72 expression

produced by short (27.2 minutes) and longer duration (58.9 minutes) trials conducted at 60 and 75% $\dot{V}O_{2\ max}$. The similar post exercise eHSP72 values were obtained despite very different mean and peak T_{core} (39°C and 39.7°C). Taken together these results would indicate that a minimum threshold of physiological and thermal strain, or "minimum endogenous criteria", is required to lead to the releases of eHSP72. It has been demonstrated that T_{core} (Ruell et al., 2006; Periard et al., 2012; Gibson et al., 2013) and aerobic capacity (Selkirk et al., 2009) are endogenous factors, which are related to the degree of eHSP72 release.

In summary, exercise induced changes in eHSP72 are intensity (Fehrenbach et al., 2005; Periard et al., 2012) duration (Periard et al., 2012; Fehrenbach et al., 2005) and mode specific. Plasma derived eHSP72 values are higher than those obtained from serum and are recommended for future use (Whitham and Fortes, 2008). Both hyperthermia and exercise alone leads to increases in eHSP72, but the combination results in the greatest response (Whitham et al., 2007; Gibson et al., 2013). Heat acclimation appears to reduce basal eHSP72 and attenuate the response to subsequent heated exercise. However the use of serum samples (Marshall et al., 2006; Yamada et al., 2007; Sandstrom et al., 2008) despite empirical data showing the benefits of using plasma samples may have affected the sensitivity of measurements in these studies. Both the origin of eHSP72 and its physiological relevance remain unclear and require further study

2.5.4 Human HSP72 response to hypoxic stress

It has been hypothesised that HSPs may play a role in the defense of hypoxic injury (Kiang et al., 1996), with such cytoprotective effects against hypoxic challenge well documented *in-vitro* within animal and human tissues/cells (Patel et al., 1995; Kiang et al, 1996; Wang et al., 2006). Animal models have confirmed HSP72 upregulation following hypoxic exposure

(Kiang et al., 1996). Despite this, this particular area of investigation remains largely unexplored within the literature regarding *in vivo* human experiments.

Disturbances to redox balance are likely to provide a signal for adaptation and increased resistance to cellular stress (Jackson, 2007; Powers, 2008; Kalmer and Greensmith, 2009; Powers, 2010). Hypoxic stress is well known to disturb redox balance *in vivo* (Bailey et al., 2000; Bailey et al., 2001) and has been shown to increase HSP72 in animal tissues (Das et al., 1995; Weinstein et al., 2004). To date only a single study has investigated the PBMC HSP72 response to acute normobaric hypoxic exposure in humans (Taylor et al., 2010). This study exposed 12 males to a 75-minute resting period during which they breathed a normobaric hypoxic gas (F_IO_2 0.14), assessing mHSP72 immediately upon exiting the stimuli, with regular samples over the 12 hours after stress exposure. This level of hypoxia has only mild affects on SpO_2 (reported values of 90-92%) and heart rate (increases of 4-8 beats•min^{-1}). This mild hypoxic challenge induced post exercise in mHSP72 of 20%, which remained elevated at all-time points in comparison to controls (Taylor et al., 2010). Thus the increase in HSP72 demonstrated post hypoxia within animal muscle/cell isolates (Das et al., 1995) appear to be present in humans *in vivo*. Hypoxia also induced significant increases in plasma TBARS, a secondary marker of oxidative stress. The authors postulated that increases in ROS production may be the signal inducing HSP72 accumulation when under conditions of hypoxia (Taylor et al., 2010). ROS are key signaling molecules for many hormones, cytokines and growth factors (Allen and Balin, 1998 Maurik, 2002) and also play a role in the activation of HSF1 (Powers et al., 2010). ROS, including those generated by hypoxia, are known to damage membrane structures and proteins, while initiating apoptotic pathways (Semenza, 2000; Kulkarni et al., 2007), each established inducers of HSP72 (Garrido et al., 2001; Bienemann et al., 2008), as are periods of reperfusion (Horowitz et al., 2007). As the

final blood sample in Taylor's study (2010) was drawn upon removal from the environmental stressor it is unclear whether the hypoxic generation of ROS as part of normal metabolism, or if the subsequent respiratory burst once removed from the environment-induced mHSP72 (Taylor et al., 2010). The HSP72 responses to hypoxic exercise in humans remain unrepresented in the literature and the effects of hypoxia on eHSP72 *in vivo* in humans has not been investigated. It is likely that the combination of exercise and hypoxia, and concomitant increase in both ROS production, and metabolic heat generation, would be a potent stimulus for HSP72 accumulation.

2.5.5 Heat shock proteins and acclimation to acute heat and hypoxia

In addition to the physiological adaptations to both heat and hypoxia outlined in Section 2.3, many changes also occur at the cellular level which are vital for the achievement of acclimatory homeostasis. In his 1997 review entitled "Heat shock proteins and heat adaptation of the whole organism" Pope Mosely speculated that heat adaptation may require the induction of the HSR and HSPs, noting that organisms naturally adapted to heat possess significant HSP accumulation (Mosely, 1997). This theory was then tested in an established rodent acclimation model. Data showed that 30 days of heat acclimation elevated HSP levels in the hearts of both rats (Maloyan et al., 1999) and mice (Broomberg and Horowitz, 2004), suggesting that heat acclimation, similar to evolutionary adaptation to hot environments, produces changes to the workings of the HSP system. It has since been established that HSPs and HSP defense pathway plays an essential role in the heat acclimation repertoire (Horowitz 2012).

During the early phase of acclimation HSPs may confer a rapid thermotolerance, then, once acclimation homeostasis has been achieved, an altered threshold for HSP production, or their

elevated basal level, may buffer the intensity of other cellular responses (Maloyan et al., 1999). Several effects of heat acclimation on HSPs have been reported. These include a hastening of the transcriptional response, which increases cellular reserves of HSP72, altering the magnitude of HSP expression upon exposure to later stress (Maloyan et al., 1999). These changes likely represent a 2-component response, with the increase in basal HSP reserves suggesting cells are endowed with "on call" cytoprotective effectors without the need for *de-novo* synthesis. Alongside this, the faster transcription rate of HSPs also serves to improve the renewal rate of HSP reserves (Horowitz, 2010). The effects of heat acclimation on the heat shock machinery has received limited attention in whole body humans, presumably due to the cost and difficulty in recruiting volunteers to implement invasive methodologies.

The effect of heat acclimation on intracellular HSP72 was first addressed by Yamada et al., (2007). In this investigation, 8 participants walked or ran (depending on individual fitness) for two 50-minute periods separated by 15 minutes of rest at 56% $\dot{V}O_{2\ max}$ over 10 consecutive days. PBMCs were analysed for HSP72 via the SDS-PAGE technique. Alongside the typical physiological measures confirming heat acclimation (decreased exercising HR and T_{core}, increased sweat rate, and expanded plasma volume), basal HSP72 was elevated on day 6, with no further increases occurring from day 6 to day 10. Post exercise HSP72 also did not change from rest on these days. Similar results were later reported by McClung et al., (2008), with basal HSP72 increasing by 18% prior to the 10th day of acclimation. These authors further examined the stress responsiveness of PBMC derived HSP72 by exposing cells to an *ex-vivo* heat shock (43°C, 1 hour, 5 hours recovery at 37°C) before and after the acclimation period. Heat acclimation attenuated the heat shock induced increases in HSP72 (3.3 fold increase on day 1, 2.2 fold increase on day 10). Taken together, these results support the observations taken from animal models of heat acclimation

regarding alterations in baseline HSP72. These constant load protocols also point towards a threshold level of strain being required to induce the HSR, limiting any further increases in basal HSP72. As physiological strain is reduced later in the acclimation period, so is the stimulus for HSP72 induction. This hypothesis was addressed using a controlled hyperthermia technique, in which T_{core} was elevated 1°C from rest over a 30-minute period, and then maintained for another 30 minutes over 11 days (Magalhaes et al., 2011). The advantage to this method is that the same elevation in T_{core} is achieved on each day of acclimation, producing a sustained and continual thermal stimulus (Fox et al., 1961, 1964; Cotter et al., 1997; Patterson et al., 2004). This sustained daily physiological strain elicited the largest increase in basal HSP72 (≈300% increase). Participants in this study performed a standardized heat stress test (HST; 90 minutes at 50% $\dot{V}O_{2\ max}$, 40°C, 45%RH) before and after the acclimation period. A ≈200% increase in HSP72 was observed following the initial HST. However, basal HSP72 was ≈ 100% greater than this post-exercise value after the acclimation period, and was not affected by the second HST. This lends support to the idea that the cellular stress required to induce HSF1 activation would need to be higher post acclimation.

The role of the HSR in conferring heat acclimation and thermotolerance in humans was recently investigated by utilizing quercitin, a potent HSF inhibiting flavanol. This compound has been shown to interfere with the HSR at the level of phosphorylation (End et al., 1991), trimerization in the cytosol (Li et al., 1999), entry into the nucleus (Jakubowicz et al., 2005), mRNA expression (Hosokawa et al., 1990; Dokladny et al., 2008) and protein accumulation (Dokladny et al., 2010), and as a result blocks the acquisition of thermotolerance (Hosokawa et al., 1992). Participants underwent two phases of acclimation during which either placebo or quercitin supplementation was administered, separated by a 3 month wash out period. The

authors reported a quercitin-induced disruption in the normal cellular accumulation of PBMC HSP72 post acclimation. This blunting of the HSR and cellular HSP72 accumulation also coincided with increased HR, T_{core}, T_{skin}, T_{body} and PSI during the post acclimation heat stress test. This would seem to indicate that the activation of HSF1 and accumulation of HSP72 is a necessary component of human heat acclimation. These results are remarkably similar to those observed in rats undergoing heat acclimation with HSP72 blockade. In comparison to controls, these animals exhibited decreased heat endurance and increased tissue damage in response to later thermal challenge (Horowitz, 2007).

In contrast to these findings, Hom et al., (2011) reported no significant increases in lymphocyte HSP72 following an 11-day heat acclimation protocol, despite participants showing the classical physiological adaptations. The authors suggest that basal HSP72 does therefore not reflect whole body acclimation status. However a closer inspection of the data reveal a large intra-subject standard deviation, which may have masked the obvious trend for increased basal HSP72 on days 10 and 11 of acclimation. It is also not clear whether subjects completed the exercise bouts at the same time of day during collection of PBMCs for HSP analysis. This is important due to the known, consistent and reproducible circadian rhythm in HSP72 expression (Taylor et al., 2010). HSP72 values are known to be at their nadir early in the morning (≈5am), and greatest in the early evening (≈5pm), therefore it is important when looking for intervention-mediated changes in HSP72, to maintain tight controls over the time of day participants complete their trials. The authors report that subjects arrived to the laboratory "between 0600 and 1000 hours" for each of the 11 days of acclimation. It is possible then that acclimation mediated alterations in basal HSP72 may have been masked by the natural diurnal variation if participants did not complete the testing sessions in which HSP72 was assessed at the same time of day. This study also utilized a lower level of ambient

temperature compared to those in which HSP72 increases were noted. Subsequently, mean peak T_{core} and HR were lower in comparison to other studies (38.48°C and 158bpm). It is possible the study of Hom et al., (2011) failed to induce the threshold level of hyperthermia (suggested to be >38.5°C; Gibson et al., 2013), and cardiovascular strain (Periard et al., 2013) required to induce the HSR.

2.5.6 eHSP72 and human heat acclimation

While the responses of eHSP72 to acute exercise bouts have received much attention, the acclimation-induced changes in eHSP72 have only been assessed by 5 studies, with contrasting results. No change in serum HSP72 was f found following 10 days of heat acclimation, although a large inter-individual difference in response may partly account for this (Yamada et al,. 2007). In a small case study (n = 1) blood samples were drawn before and after hyperthermic exercise (32°C, humidity not reported) for 15 consecutive days (Sandstrom et al., 2008). During the initial 3 days of acclimation resting plasma HSP72 increased, while post exercise values were depressed from day 2 onwards, indicating a potential desensitizing of the eHSP72 response. This study also reported an inverse relationship between resting and post-exercise eHSP72 (Sandstrom et al., 2008). In direct contradiction to this, eHSP72 levels have been shown to decrease over the initial early phase of heat acclimation (<3 days; Marshall et al., 2006), at the end of 5 days of heat acclimation, but only in participants displaying classical acclimatory adaptations (reduced T_{core} and HR; Kresfelder et al., 2008) and on the final day of a 10-day acclimation period (Magalhaes et al., 2010). Although these projects have reported peak increases in eHSP72 of 90% (Marshall et al., 2006; Yamada et al., 2007; Sandstrom et al., 2008), these are small when compared to thermoneutral exercise bouts utilizing EDTA plasma HSP72 for analysis (Whitham et al., 2006). The use of serum-derived samples has persisted despite an empirical based data set

supporting a project wide switch to EDTA plasma sampling. These issues with sample collection make data comparisons between multiple studies difficult. As the biological role of eHSP72 nor its release pathway are not yet known, the significance of either an increased (Sandstrom et al., 2008) or decreased (Marshall et al., 2006; Kresfelder et al., 2008; Magalhaes et al., 2010) amount of the circulatory proteins is not known.

2.5.7 PBMC and eHSP72 responses to hypoxic acclimation

To the authors' knowledge, there have only been 2 experiments investigating repeated hypoxic exposure on basal mHSP72 expression in humans. During each investigation participants were exposed to a 75-minute resting daily hypoxic stress (2980m) for 10 (Taylor et al., 2011) or 5 (Taylor et al., 2012) consecutive days. These studies utilized a highly sensitive flow cytometric analysis technique to determine monocyte specific HSP72 (mHSP72). They observed a 30% daily increase in basal mHSP72 for the initial 5 days of exposure. Thereafter a plateau in response was noted with ≈16% day on day increases between days 6 – 10. This total ≈ 200% increase in mHSP72 is similar to that observed following exercise-heat acclimation (Magalhaes et al., 2011), though the different tissues examined make a direct comparison difficult. Later, a 5-day period of identical resting hypoxic exposure induced a comparable increase (≈60%) in basal mHSP72, which persisted for 48 hours after the final hypoxic session (Taylor et al., 2012). This suggests that factors independent of changes in T_{core}, such as increased oxidative stress and reactive oxygen species formation, also contribute significantly towards HSP72 accumulation. There appears to be no data regarding the eHSP72 response to hypoxia, either acutely or after repeated sessions. A summary of studies examining the intracellular and extracellular HSP72 responses to acclimation (both heat and hypoxia) in humans are shown in Table 2.2.

2. 6 Preconditioning and cross-acclimation

The previous sections have focused on both the acute physiological responses to heat and hypoxic stress, and the typically observed adaptive responses to each stressor following prolonged, or repeated exposures. It is clear that each stressor delivers unique challenges to the maintenance of homeostasis. However each of these stressors appear to set in motion a general pattern of physiological and cellular responses. It is these shared responses that give rise to the possibility of cross-acclimation existing between heat and hypoxia. There is now a substantial body of evidence showing that heat acclimation can induce a positive cross tolerance against other environmental stressors associated with altered oxygen, such as hypoxia, ischemia-reperfusion, hyperoxia, and traumatic injury (Levi et al., 1993; Arieli et al., 2003; Paz et al., 2004. This section will outline research into acute preconditioning studies between hyperthermic stress and hypoxia, and then consider the longer-term cross-acclimation literature.

Table 2.2. Summary table of human studies investigating intracellular HSP72 (iHSP72) and extracellular HSP72 (eHSP72 responses to heat or hypoxic acclimation.

Authors	Acclimation protocol	Tissue	Measurement	Results (HSP72)
Heat/icHSP72				
Yamada et al., 2007	10 days walk/run 100-mins 53% $\dot{V}O_{2\ max}$, 42.5°C.	PBMC	SDS-PAGE	↑ 100% days 6-10 compared to control
Marshall et al., 2007	Cycling 120-mins 38% $\dot{V}O_{2\ max}$ (38°C) 3 consecutive days.	PBMC	RT-PCR	No significant ↑ HSP72 protein at any time point.
McClung et al., 2008	Treadmill walking max 100mins per day, 1.56m/s 4% gradient 10 consecutive days 49°C	PBMC	Western blot	200% mRNA post exercise day 1 18% ↑ HSP72 protein on day 10
Magalhaes et al., 2010	90-mins treadmill running 50% $\dot{V}O_{2\ max}$ (40°C) pre (HST1) / post (HST2) 11 day acclimation period (30 min treadmill running 1% grade, 7.9km•hr^{-1}, 30 min treadmill running 1% grade 6.0km•hr-^{1}.	PBMC	Western blot	Pre to post HA ↑300% HST1↑ 200% (post), ↑350% (1h post) HST2↑300% pre post and 1h post compared to control.
Amorim et al., 2011	10 days, walk/run, 50% $\dot{V}O_{2\ max}$, 42.5°C.	PBMC	ELISA	Pre to post HA resting HSP72
Hom et al., 2011	Treadmill walking 50% $\dot{V}O_{2\ max}$ at 5.6km·hr^{-1}, 5% grade (33°C, 30-50% RH) 11 days.	PBMC	Flow cytometry	No ↑ HSP70 +tive cells from day 1 to 11. ↑ HSP70 MFI on day 10 relative to day 1

Kuennen et al., 2011	45mins walking at 50% $\dot{V}O_{2\ max}$ (HST) with and without quercitin supplementation. HA 50mins walking (46.5°C) to increase T_{core} to >39°C. 10 mins rest, 50mins walking with T_{core} >39°C. Acclimation procedure performed with and without quercitin supplementation	PBMC	Western blot	No significant ↑ HSP72 after both HST. 75% ↑ resting HSP72 following HA (placebo) No change in resting HSP72 following HA (quercitin).
Taylor et al,.2011	75 minutes resting hypoxia F_IO_2 0.14 for 10 consecutive days	PBMC	Flow cytometey	30% day on day basal ↑ until day 5. ↑ basal mHSP72 ~16% from day 6 – 10.
Taylor et al., 2012	75 minutes resting hypoxia, F_IO_2 0.14 for 5 consecutive days	PBMC	Flow cytometry	30% day on day basal ↑ in mHSP72
<u>eHSP72</u>				
Marshall et al., 2006	3 days cycling, 2 hours, 40% $\dot{V}O_{2\ max}$, 38°C	S	ELISA	50% ↓ on day 3
Kresfelder et al., 2006	5 days, 60-mins step test, 35 Watts for 15 mins, 70 Watts 15-mins, 28°C	S	ELISA	20% ↓ after 5 days
Yamada et al., 2007	10 days walk/run 100 min 53% $\dot{V}O_{2\ max}$, 42.5°C	S	ELISA	110% ↑ post acclimation
Sandstrom et al., 2008	15 days cycling at 50% $\dot{V}O_{2\ max}$, 32°C.	S	ELISA	110% ↑ after 15 days
Magalhaes et al., 2010	90 mins treadmill running 50% $\dot{V}O_{2\ max}$ (40°C) pre (HST1) / post (HST2) 11 day acclimation period (30 min treadmill running 1% grade, 7.9km•hr^{-1}, 30 min treadmill running 1% grade 6.0km•hr^{-1}.	P	ELISA	No change pre to post acclimation. 40% ↑ post HST1 No change post HST2

2.6.1 Preconditioning and the first and second windows of protection

The discovery that four periods of coronary ischemia (5-minutes), interspersed with 5 minutes of reperfusion immediately before occlusion of the circumflex artery for 40mins, reduced infarct size in dogs by 75% was a seminal discovery that has enhanced our understanding of cell survival machinery (Murray et al., 1986; Yellon et al.,2003). This phenomenon, termed ischemic preconditioning (IPC), represents a ubiquitous endogenous stress response, which has been subsequently reproduced by numerous laboratories and in multiple species, including clinical trials in humans (Hausenhloy et al., 2005). The PC effect has been observed in heart, kidney, liver and brain (Yellon and Downey, 2003). This method has been described as the strongest form of protection against myocardial ischemic injury (Kloner et al., 1998). Since its initial discovery over 8200 papers have been published on the topic (May 2014, Pubmed, search term "Ischemic preconditioning").

The initial stress exposure which constitutes the PC stimulus has been found to elicit two separate protective windows for cardioprotection: the first window, termed classical preconditioning (CPC) is evident immediately, and is transient in nature, with the level of protection subsiding after 2 – 3 hours (Hausenhloy and Yellon, 2009). Approximately 12-24 hours after the initial PC stimulus, a second window of protection (SWOP), which lasts between 2 – 3 days (Marber et al., 1993). This second window of protection requires new RNA and protein synthesis, and the activation of a host of pathways, with the two most important pathways related to HSPs, and hypoxia inducible factor - 1 alpha (HIF1-α).

2.6.2 Hyperthermic and hypoxic preconditioning

Transient exposure to environmental stressors, usually hyperthermia, has also been shown to convey protection to subsequent related and non-related stressors. This protective effect is, in part, underpinned by changes in the concentrations and expression kinetics of HSP72 (Morimoto and Santoro, 1998). The effect of hyperthermic PC has also been shown to be beneficial upon subsequent exposure to a multitude of stressors, limiting negative outcome to subsequent lethal and sub-lethal insults (Kalmer and Greensmith, 2009).

Classic PC investigations utilize experimental models in which cells are heat shocked with a sub lethal challenge designed to induce HSP72 at the protein level. After approximately 24 hours stressful exposures can then be counteracted more efficiently than compared to naïve, non-heat-shocked cells (Zhang et al., 2002). This form of PC has been shown to be effective in improving whole body exercise tolerance to acute heat (Chen, 1999). In this study animals underwent a 15-minute whole body heat shock at 41-42°C 2 days prior to completing an exercise bout in hyperthermic conditions (36.5°C). The authors reported that the heat-shocked animals were able to exercise significantly longer than control animals (89 vs 63 minutes). The improved exercise capacity, was in part, attributed to significant elevations in skeletal muscle and leukocyte HSP72.

An early study investigated the relationship between the degree of myocardial HSP72 induction and the degree of protection from subsequent ischemic injury (Hutter et al., 1994). Female Sprague-Dawley rats were exposed to 4 levels of whole body hyperthermia: control (no treatment), 40°C, 41°C, and 42°C for 20 minutes.

Following a 24-hour recovery period, small myocardial samples from the anterior left ventricle were harvested. Animals then underwent a 35-minute left coronary artery occlusion, followed by 120 minutes of reperfusion. The authors reported a strong inverse correlation (r = 0.97, P = 0.037) between the amount of cardiac HSP72 induced by prior heat shock and infarct size after subsequent ischemia reperfusion. Utilizing transgenic approaches to upregulate HSP72 expression in mice further elucidated the importance of HSP72 to myocardial protection. This classic research demonstrated a resistance to the biochemical rigours of ischemia reperfusion injury. These HSP72 overexpressing animals displayed a favourable return to optimal myocardial contractile function, and reduced myocardial damage compared to control animals, which had a lower expression of HSP72 (Marber et al., 1995; Pumier et al., 1995). The positive effect of heat shock and the parallel increase in basal HSP72 has been shown to augment reactive oxygen species (ROS) scavenging ability, and reduce muscle damage following downhill running compared to control animals (Shima et al., 2008).

Heat shocked cells, when exposed to a subsequent stressor *in vitro* demonstrate a blunted HSP72 in comparison to the initial response to the primary stressor (Li et al., 1983; Meyer et al., 1983; Ryan et al., 1991). This blunted HSP response to *in vitro* heat shock has also been observed in human PBMCs following exercise (Ferenbach et al., 2000). Both *in vivo* and *in vitro* research has shown that the increases in post stressor HSP72 is directly proportional to the basal content in the tissues examined. Furthermore, within exercising humans, a prior exercise bout blunted both the mRNA and protein HSP72 responses to a repeated identical exercise bout (Vissing et al., 2009). It is possible that the further translation of HSP72 in response to a later stressor

is suppressed via negative feedback due to the already high levels of HSP72 present. This would represent an increase in the threshold stress required to separated the HSP complex from HSF1 to initiate further production (Madden et al., 2008).

Hypoxic preconditioning has also been shown to be a potent PC stimulus in rodent models. There is a tendency within the literature to confuse hypoxia with ischemic PC, which may be related to the degree of hypoxia employed. If animals are exposed to 8 or 9% oxygen they may develop cardiac arrhythmias and systemic hypotension and ischemia that may induce genes related to the systemic ischemia and not due to the hypoxic stimulus *per se*. Thus for hypoxic PC to be securely investigated it is essential to ensure maintenance of cardiac rhythm and blood pressure (Gidday et al., 1994; Miller et al., 2001; Bernaudin et al., 2002). When cardiac rhythm and blood pressure effects are properly controlled for, the exposure of neonatal rat pups to hypoxia alone (F_IO_2 = 0.08 for 3 hours) protected these animals 24 hours later from a stroke induced by combined hypoxia/ischemia using carotid occlusion and exposure to 8% oxygen for 3 hours (Gidday et al., 1994). A finding that was later repeated in adult rodents (Miller et al., 1999). Later, mice exposed to a period of whole body hypoxic preconditioning (F_IO_2 = 0.08 alternated with room air for 6 x 10 minute cycles) showed increased survival times when exposed to lethal hypoxia 2 hours later (F_IO_2 = 0.07; Zhang et al., 2004).

It is likely that hypoxic PC is partly regulated by the induction of HIF-1α. This has experimental support from both whole body animal studies, and pharmacological interventions, both of which induce HIF-1α and its associated targets. For example, neonatal rats exposed to 8% O_2 for 3 hours induced HIF-1α in the rat brain, alongside

target genes EPO and VEGF (Kapinya et al., 2002; Bergeron et al., 1999; Bernaudin et al., 2002). Pretreating animals with pharmacologic HIF-inducing compounds (cobalt or disferrinoxamine) protected the brain against a later ischemic injury (Ratcliff et al., 1998; Semenza et al., 1999; Mole et al., 2001; Maxwell and Radcliffe, 2002).

In summary, PC responses to acute stress exposure and the mechanisms that drive those responses are well described in animal tissues and species, there has been very little research in whole body human models when relating to exercise performance. It can be seen that heat-shocking cells with non-lethal thermal stress confers a rapid cytoprotective influence, in part mediated by an up-regulation in HSP72. This up-regulation exerts anti-apoptotic effects, which improve cellular tolerance (decreased HSP72 transcription) in response to exposure to later stressors as the previously elevated HSP72 content increases cellular protection (Madden et al., 2008). This represents an increase in the threshold stress required to initiate further HSP72 transcription. The upregulation of HIF-1α and many of its target genes plays a role in the hypoxic-mediated PC response, however this has not been investigated in humans. It is surprising that little research exists in whole body human models regarding the potential PC effects that accessible environmental stressors, such as acute heat exposure, may provide when later presented with challenges to oxidative metabolism, such as hypoxia or strenuous exercise. It is possible that shared molecular signalling responses between heat and hypoxia play some role in the PC and cross-tolerance response. This is explored in Section 2.7.

2. 7 Heat acclimation mediated cross-tolerance

In addition to the PC effect previously described (Section 2.6), there is evidence that longer-term heat-acclimatory adaptations also induce protective effects when exposed to stressors related to altered oxygen supplementation (Levi et al., 1993; Ariel et al, 2003). It is likely that the cross-tolerance/cross acclimation effects emerge from alterations in the capacity or responsiveness of molecular signaling pathways shared by the primary (heat) and secondary (hypoxia/ischemia) stressor. The majority of research examining heat acclimation mediated cross-tolerance has predominantly been conducted in rodent models within the Horowitz laboratory in Israel. Therefore this section will provide a brief overview to heat-acclimation cross tolerance studies, and the cellular mechanisms mediating such effects.

2.7.1 Cross acclimation in animals

Acclimation-mediated cross acclimation differs from PC and SWOP because rather than providing improved tolerance for hours and days, the effects of acclimation can be seen on secondary stressors for weeks. This makes studying both short term and longer term PC and acclimation protocols important. The earliest mention of heat acclimation mediated tolerance to an oxygen supply stressor was by Hiestand et al., (1955). These authors observed heat acclimated rodents better survived drowning than their non-acclimated counterparts (Hiestand et al., 1955). This early finding was correlated to reduced thyroxine levels observed in animals. Later, intergrative studies revealed the hemodynamic recovery of HA hearts from ischemia/reperfusion (I/R) or hypoxia was enhanced in the HA animals (Levi et al., 1993). Typically the HA protocol for these studies exposed animals to a 32°C environment constantly for 30 days, so drawing comparisons with human acclimation studies is difficult. For

example, acclimated animals display a greater time to the onset of ischemic contracture compared to control animals (8 ± 2 minutes compared to 6 ± 1.3 minutes), a markedly greater diastolic recovery and ventricular filling pressure recovery post I/R (80% compared to 35%) and a greatly reduced infarct size and area at risk (4.3 ± 1% compared to 30 ± 7%) (Levi et al., 1993; Levi et al., 1997; Horowitz et al., 1997). These improvements in cardiac function were related to an enhanced anaerobic ATP generation occurring at a slower rate, which favors intracellular pH regulation, and decreases ATP utilization during global ischemia (Levi et al., 1993; Enyan et al, 2002). Whole body HA has also been shown to be protective during whole body hypoxic exposure (14.5% PO_2 for 15 minutes), as indicated by improved cognitive function in rodents (Horowitz, 2007). Although this is a well-established phenomenon in animal models there is little human research to draw upon regarding heat-mediated tolerance to oxygen deprivation stressors.

Research examining living at high altitude while training at sea-level is abundant within the literature, and has shown that this paradigm may improve performance upon return to sea level above that of training and living at sea-level (Stray-Gunderson et al., 1997; Millet et al., 2010). The effects of a period of heat acclimation (10 days cycling for 90 minutes at 50% $\dot{V}O_{2\,max}$ in 40°C, 30%RH) on maximal aerobic power, time-trial performance, and lactate threshold, were examined upon return to cool conditions within 1 week of the last acclimation session. This was a well-controlled study utilizing a normothermic control group (the same exercise bouts performed at 13°C), with pre and post exercise tests conducted in both normothermic and hyperthermic conditions in each group (Lorenzo et al., 2010). In comparison to controls, the HA group had improved $\dot{V}O_{2\,max}$ in both cool and hot conditions (5%

and 8%), an improved time trial performance in each condition (6% and 8%) and similar improvements to lactate threshold in each condition (5%). Heat acclimation also increased plasma volume (6.5 ± 1.5%) and maximal cardiac output in both heat and control conditions (9.1 ± 3.4% and 4.5 ± 4.6%). These data demonstrates that heat acclimation has the potential to improve performance in temperate conditions (Lorenzo et al., 2010). The improved physiological efficiency, despite the opposing adaptations to hematology, may induce a significant improvement when exercise is performed in hypoxic conditions. It is therefore surprising that to date, only one study has investigated the effects of a prior period of HA on later hypoxic performance (Helad et al, 2012).

This project examined whether the effects of 12 days of exercise HA (2 hours walking at 30% $\dot{V}O_{2\ max}$ in 40°C, 40%RH) would reduce physiological strain during a graded onset of blood lactate accumulation (OBLA) test and also affect cognitive performance in both normoxia and mild hypoxia (F_IO_2 0.156, 2400m asl), (Helad et al., 2012). The workload used for acclimation presented a mild physiological stressor, with peak HR and peak T_{core} decreasing from 115 ± 13beats•min^{-1} and 37.83 ± 0.2°C on day 1, to 103 ± 14beats•min^{-1}, and 37.59°C on day 12. Unlike Lorenzo et al., (2010), this protocol did not elicit any changes in $\dot{V}O_{2\ max}$ values. However, OBLA was increased in both conditions, evidenced by higher HR measured at OBLA. SpO_2 tended to be higher during the hypoxic test after HA, however the raw data are not presented in the manuscript. The authors suggest that the mild effects on physiological efficiency post HA, in the absence of improved aerobic power, strengthens the hypothesis that heat per se, and not a training effect, is the main contributor to the increased physiological efficiency observed in normoxia and

hypoxia (Helad et al., 2012). Mild, likely trivial, improvements in cognitive function, as assessed via four-choice-reaction-time (FCRT) and visual vigilance task (VVT) were also observed under hypoxic conditions post HA. Although this study employed a familierisation period prior to baseline cognitive tests, the experimental protocol did not employ a counterbalanced design, thus all participants completed normoxic tests, and 24 hours later, hypoxic tests pre and post acclimation. A learning effect between the baseline tests, and post acclimation tests cannot therefore be discounted for these measurements. The authors speculate that HA may have induced the cytoprotective machinery known to be protective following acclimation in animal models. However this would seem unlikely as the physiological strain imposed by their acclimation model is not of the magnitude required to induce, for example, HSP72 in humans (i.e. HR >160beats•min^{-1} and T_{core} >38.5°C) (Selkirk et al., 2009; Periard et al., 2012; Gibson et al., 2013).

In summary, the cross-tolerance effects of HA and oxygen deprivation stressors are well defined and consistently observed in rodent models (Levi et al., 1993; Levi et al., 1997; Horowitz et al., 2004; Maloyan et al., 2005), with heat acclimation seemingly inducing multiple adaptive changes at the level of the myocardium. This protective effect of HA has received scant attention in whole body humans, despite HA inducing significant improvements to physiological efficiency at sea level (Lorenzo et al., 2010). The following section will examine the molecular and cellular mechanisms behind cross-acclimation in context of both the short term, and long-term models employed.

2.7.2 Mechanisms of cross acclimation

The cellular components and signaling pathways involved in the attainment of the HA phenotype, and subsequent cross-tolerance effects, have been thoroughly investigated by numerous studies by Horowitz and colleagues (Horowitz et al., 2004; Maloyan et al., 2005; Tetievsky et al., 2008). These authors utilized gene chip technology to piece together a detailed stressor-related genomic response profile for the transition between STHA and LTHA in order to test the hypothesis that HA induced tolerance to oxygen deprivation stressors is coupled with reprogramming the expression of genes which alter the responsiveness of signaling pathways shared by heat and ischemia (Horowitz et al., 2004). Differences in gene profiles were collated for control animals; control animals exposed to a later heat shock; control animals later exposed to I/R insult, HA animals (30 days); HA animals exposed to a later heat shock, and HA animals exposed to a I/R insult. This allowed the authors to examine to what extent heat shock and ischemia reperfusion induce similar and/or different genes, as well as determine how HA influences later gene responses to I/R.

The authors found 4 clear gene response profiles associated with these stressors.

- genes of which HA only effects basal levels
- genes of which HA only effects the response to acute heat shock
- genes of which HA only effects both basal steady state levels, and the response to stress
- genes responding to HA and HS independently.

Within the first category, were genes assigned to maintaining DNA integrity, free radical scavenging, and stress effectors and regulators. Many genes in this group showed only meaningful expression during the STHA phase, with only faint detection

upon acclimation and LTHA. The majority of these genes were not affected by acute HS, which suggests that sustained heat strain, as seen over the course of an acclimatory period, is required for their induction (Horowitz et al., 2004). The genes in clusters 2 and 3 described above responded to heat stress with an elevated transcriptional level compared to the non-acclimated animals. The majority of these genes were stress response effectors and regulators. Their activation possibly enhances a HA specific cytoprotective signaling network to cope with any disruptions to homeostasis after LTHA. The final category contained the chaperone genes (e.g. HSP72, HSP90). An interesting observation was that acute heat stress increased transcription of these genes 4-fold irrespective of the acclimation phase, suggesting that HS and HA induce two distinctive responses. These results also support the notion of a two-tiered adaptive profile seen during the physiological aspects of heat acclimation. Most of the I/R responsive genes were independent of those observed during HA.

The shared gene transcription response to heat acclimation and ischemic stressors was previously shown to be the case in human hepatocytes exposed to either an acute heat shock, or hypoxic stressor (Sonna et al., 2004). These authors reported a small overlap in gene expression between the two stressors stressors (11-22%). An important factor in the cross tolerance response may be related to the earlier threshold for gene activation upon stress exposure, especially in regard to the HSP network. The greater responsiveness of HSPs post acclimation had earlier been noted (Maloyan et al., 1999), and was here found to reach peak responsiveness at 50% ischemia in HA hearts compared to non-acclimated animals. Thus, this universal role of HSP72 in the stress response, coupled with its faster transcription following HA, indicates that

cross-acclimation involves the reinforcement of cytoprotective pathways (Horowitz et al., 2004).

The ubiquitous response of the cytoprotective pathways further demonstrates the importance of HSPs as protective guardians during both heat and I/R challenge. However, the observation that heat acclimated animals also display enhanced glycolysis, greater glycogen sparing, and elevated levels of GLUT-1 and GLUT-4, led researchers to investigate the role of HIF-1α in heat acclimatory processes and cross tolerance (Trein et al., 2003). That HIF-1α plays a major role in the adaptive cascade of responses to O_2 deprivation, regulating genes associated with oxygen and energy metabolism (e.g. erythropoietin), and mediates adaptation to chronic hypoxic exposure (Semenza, 2004; Sham et al., 2004), it is highly likely that it also plays an important role in any HA metabolic response. The importance of HIF-1 α to the attainment of HA was first examined in a HIF knockout nematode model of acclimation. HIF-knockout *Caenorhabiditis elegans* was found to be unable to adapt to heat, showing this transcription factor is essential for obtaining acclimatory homeostasis (Trein et al., 2003).

The role of this transcription factor in HA was further addressed by an intricate set of experiments which aimed to characterize the expression kinetics and transcriptional activation of HIF-1α during HA, whilst also elucidating its role in cross-tolerance (Maloyan et al., 2005). To characterize the effects of heat stress on control and HA rats, the animals were exposed to a heat shock challenge (41°C, 2 hours) and allowed to recover for 0, 30 and 60 minutes prior to sacrifice. Cross-tolerance was assessed by exposing the hearts of HA and non-HA animals to a reduction in perfusion pressure

(50, 75 or 100% global ischemia) for 45 minutes. To determine any differences in ischemic injury, hearts from HA and non-HA animals underwent 30 minutes of global ischemia followed by 30 minutes of reperfusion, with infarct size determined by the area at risk. A 203% increase in myocardial HIF-1α was found in HA animals, and remained unchanged in control animals. The non-HA rats exposed to a heat shock displayed elevated HIF-1α for at least 1 hour after recovery. In contrast, in the HA hearts heat stress did not increase HIF-1 protein above that already observed post HA. The functional role of HIF-1α following HA was confirmed by increased transcription of VEGF. Additionally, both EPO gene and EPO-receptor (EPO-*r*) transcripts were shown to be upregulated in response to HA, which was maintained upon exposure to I/R (Maloyan et al., 2005). These increased levels of HIF-1α and EPO-r mediate the protective function of erythropoietin by targeting metabolic and anti-apoptotic cascades. This has the effect of ensuring the HA heart is protected and provides a molecular framework for HA cross-tolerance mechanisms (Maloyan et al., 2005). It was also noteworthy that EPO was upregulated in the kidney, further illustrating the global effects of HA. HSP72 protein is observed in the brain of HA rats, and higher HIF-1 α and EPO-r in HA mice brains, has also been observed, further supporting these conclusions (Oppenheim et al., 1996; Arieli et al., 2003; Shein et al., 2005).

In summary, these data show that enhanced basal levels of cytoprotective proteins, and their more rapid response following HA, are important mediators of cross-tolerance. Since both HSPs and HIF-1α are key regulators of heat, hypoxic and ischemic preconditioning responses, it is no surprise that they also play a role in HA and cross-tolerance. This is a feature of acclimation yet to be conclusively studied in whole body humans. The observation that both acute and chronic heat or hypoxia

induces increases in basal HSP72 gives rise to the possibility that adaptation to heat may improve both cellular and whole body tolerance to hypoxia. This remains an understudied area in human physiology.

2.8 Overall summary

The physiological and cellular responses to acute heat and hypoxic stress present a considerable overlap both at rest and during exercise (Figure 2.1 and 2.2). Both acute heat and hypoxic exposure induce HSP72 *in vivo* in humans (Selkirk et al., 2009; Taylor et al., 2010). However research examining both the physiological and HSP72 responses to these stressors alone and in combination within the same cohort of participants is lacking. The first phase of this thesis addresses this area. Establishment of both the physiological and cellular responses to heat, hypoxia, and the combination thereof is an important first step in exploring pre-conditioning and cross-acclimation (Fregly, 1996).

Classic physiological signs of heat acclimation are a reduced core temperature and heart rate (Aoyagi et al., 1997). The increases in plasma volume partly account for the increased cardiac stability observed following heat acclimation (Garrett, 2011). Additionally, sweating begins at a lower core temperature threshold allowing earlier heat dissipation and maintenance of cooler skin temperatures. After 7 – 14 days of heat acclimation approximately 50 and 80% of the physiological adaptations are complete (Garrett 2011). Similarly, repeated exposures to hypoxia, in which acclimation is conferred are characterized by functional changes that restore the PaO_2. The major physiological adaptations to altitude are an increased ventilation which raises partial pressure of arterial O_2 and arterial oxygen saturation, an increased tissue

extraction of O_2 from capillary blood, increased sympathetic nervous activity which sustains blood flow and blood pressure, increased carbohydrate transport and utilization, the increased 2,3-diphosphoglycerate and renal bicarbonate excretion promoting O_2 unloading from hemoglobin, a decreased plasma volume which raises arterial O_2 content via increased hemoglobin concentration, and an erythropoietin mediated increase in red cell mass which all raises arterial O_2 content (Young and Reeves, 2002; Favret and Richalet, 2007; Mazzeo, 2008). These adaptations translate to a reduction in exercise HR for a given level of submaximal exercise and overall reductions in physiological strain for both heat and hypoxia. Interestingly, the divergent/opposite adaptations to plasma volume during these stressors may point towards negative cross-acclimation. However the improved physiological efficiency seen as a result of heat acclimation may compensate for the lack of hemoconcentration (Lorenzo et al., 2010; Helad et al, 2012).

The post exercise response of intracellular HSP72 is well documented, as is the response of eHSP72. The role of a prior induction of HSP72 is an important feature in animal models of preconditioning (Hutter et al., 1994) and cross-acclimation (Maloyan et al., 1999). However the importance of this protein in the human preconditioning response to stress has not been explored. Determining the relative importance of acute heat stress, or acute hypoxic stress in HSP72 expression has yet to be compared within participants. Any environmental stress-mediated changes in basal HSP72 expression may enable the transfer of cross-acclimation to subsequent, non-lethal stress exposure in humans. HSP72 induction is an integral aspect of the heat acclimation process in rodent models (Maloyan et al., 1999) and has also been shown to be important to the development of acquired thermotolerance in humans

(Kuennen et al., 2010). These authors demonstrated the blocking of the HSR via acute quercitin supplementation, negatively impacted on physiological strain following a period of acclimation (Kuennen et al., 2010). HIF-1α plays an important role in the development of the heat acclimated phenotype (Trein, 2003; Maloyan et al., 2005), with HIF mediated transcription of EPO and VEGF reported in heat acclimated animals (Maloyan et al., 2005; Tieteivsky et al., 2008). The increase in these proteins possible confers cytoprotective effects to the myocardium. These shared molecular adaptations between heat and hypoxia have yet to be investigated *in-vivo* in humans, and the HIF-1α response to heat acclimation has also not been examined.

2.9 Aims and purpose

The experiments within this thesis aim to establish the physiological and HSP72 responses to individual and combined heat and hypoxia at rest and during and after exercise. It is well established that acute exposures to both these stressors increase HSP72 in humans. However the relative role of heat/hypoxia in HSP72 expression has not been determined. The magnitude and persistence of this response may be different between stressors over the 24-48 hour second window of protection, thus examining alterations in basal HSP72 in the period following the initial stress exposure would provide information regarding the preconditioning and cross-acclimation potential of such acute exposure. The goal of increasing basal HSP72 would be in initiating conferred tolerance to the biochemical rigors of exercise in a hypoxic environment.

Additionally, it is known that repeat exposures to heat or hypoxia initiate adaptive responses which serve to reduce physiological and cellular strain upon later exposure

to the stressor. The adaptive responses to each of these stressors elicit similar effects on the basal and stress inducible characteristics of HSP72. Thus determining whether a prior adaptation period to heat stress offers a similar or enhanced improvement to hypoxic tolerance, compared to a time matched period of hypoxic adaptation would be of interest. Although research has shown the ergogenic potential of heat acclimation regarding sea level performance, no *in vivo* human study has investigated whether heat acclimation *per se* can improve physical performance in hypoxic conditions. The aims of this thesis are outlined at the end of Chapter 1.

CHAPTER 3. GENERAL METHODS

3. 0 General methods

The following sections describe the materials and methods common to each experimental chapter. If additional materials or modified methods were used, details are provided in the relevant section within the respective experimental chapter. All experiments took place inside Coventry University's physiology laboratories, which are maintained according to the guidance of the British Association of Sport and Exercise Scientists (www.bases.org.uk/Laboratory-Criteria).

3. 1 Ethics

The Coventry University Ethics Committee approved all investigations. Written and verbal explanations of the protocols, including the potential risks, discomforts, and benefits of taking part were given to all potential volunteers prior to recruitment. Informed consent forms were completed by participants prior to any testing. Participants confirmed that they understood the potential risks involved and that they were able to withdraw from experimentation at any time without providing a reason. Medical questionnaires were competed to confirm that they were in good health when participating, and Inclusion criteria included: no history of cardiovascular, respiratory, nervous, renal, liver, skeletal/muscular or metabolic disease; no immunosuppression with HIV or forms of medication; no recent allergic reactions or illness; normal haemoglobin (> $13 g \cdot L^{-1}$) and haematocrit (> 40%). In studies involving heat acclimation participants were recruited who had not visited hot climates for at least 2 months prior to the testing dates in order to minimise potential confounding effects of partial acclimation. All investigations conformed to the principles outlined in the Declaration of Helsinki (World Medical Association, 2000).

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3. 2 Participant care

Heart rate, oxygen saturation, and core temperature were continually monitored throughout all experimental trials according to the methods stated in Sections 3.4, 3.5 and 3.7 respectively. Participants were under constant observation such that experiments were ceased if any of the following criteria, determined from departmental ethical considerations, were met:

- the participant requested to stop;
- the experimenter decided to stop, if the participant displayed signs of discomfort, nausea, heat syncope, heat exhaustion, or anaphalactoid shock type symptoms;
- rectal temperature increased above 39.5°C, or increased more than 2°C from resting values;
- arterial oxygen saturation fell below 70% and was not immediately corrected (Thake, 2006);
- the participant exhibited symptoms or signs of dizziness, mental confusion, severe restlessness, incipient faint.

3. 2. 1 Participant preparation

Prior to reporting to the laboratory for each visit, participants abstained from alcohol, caffeine, and exercise for 48 hours and maintained their normal diet. They were asked to write down and replicate these habits/activities prior to subsequent visits. Participants reported to the laboratory at the same time of day in order to minimise the influence of circadian variation (Winget et al., 1985; Reilly, 2006) and experimentation was conducted at least 3 hours after food consumption. All testing described in Chapters 4 and 5 was performed on a Monark cycle ergometer (Monark

874e, Sweden). All testing described in Cchapter 6 was carried out on an electromagnetically braked cycle ergometer (Schroberer Rad Metechnik, Germany; Section 3.11.2).

3.3 Preliminary measures

During the initial laboratory visits each participant's anthropometric variables were assessed, followed by a continuous cycling lactate threshold and $\dot{V}O_{2\ peak}$ test Section 3.6.1). Body mass and stature were measured in the Frankfort plane using balanced weighing scales and a stadiometer (Seca, Bodycare, Southam, UK) to the nearest 0.1 kg and 1.0 cm respectively. Sum of skinfolds was measured on the right side of the participant using Harpenden callipers (British Indicators Ltd, London, UK) from the triceps, biceps, subscapula, suprailliac, front thigh and medial calf as described by Stewart and Eston (2006). Percent body fat was estimated according to Durnin and Womersley (1974). Limb girths were measured using a metal tape measure (Korbond, Chersry, Surrey, UK) at the upper arm (midway between the acromiale and radiale), thigh (midway between the inguinal crease and the midpoint of the patella) and calf (at maximum girth). An equation employing stature, thigh and calf circumferences and corrected for skinfold thickness and uncorrected forearm girth was used to estimate whole body muscle mass (Martin et al., 1990).

3.3.1 Manipulation of the external environment

All normothermic, normoxic exercise testing was conducted in an air conditioned laboratory (18°C, 30-40% RH**).** Trials involving hyperthermic conditions were conducted in a custom made heat tent (3 x 4m) warmed to the experimental temperature (~40°C, 20%RH) with a series of heaters placed throughout the room.

Temperature and humidity were constantly monitored using a heat stress meter (Kestral 4400 Heat stress meter, Kestralmeters, USA) and heaters manually adjusted to maintain a constant room temperature. Normobaric hypoxia was generated using a Hypoxicator (Hypoxico HYP123 Hypoxicator, New York, USA). The experimental altitude was maintained at 3095m asl in all experimental conditions described throughout this thesis. The hypoxic gas was delivered to participants by breathing through a mouthpiece and 30 mm diameter connector (Harvard Ltd, Eldenbridge, UK) attached to a two-way non-rebreathable valve (Harvard Ltd, Eldenbridge, UK). Ethylene clear vinyl tubing was used to connect the inspiratory side of the valve to a series of 1000L Douglas bags used as a hypoxic gas reservoir. The Douglas bags were checked for leaks on a regular basis and the F_IO_2 of the filled Douglas bags was also monitored on a regular basis to ensure consistency.

3. 4 Cardiorespiratory measurements

3. 4. 1 Heart rate

Heart rate (Suunto, T6c) was continuously monitored throughout each trial using short-range telemetry and the data were analysed using Suunto Training Manager software.

3. 4. 2 Arterial haemoglobin saturation

Arterial haemoglobin oxygen saturation (SpO_2) was continuously monitored in all experiments via an earlobe sensor and pulse oximeter (Nonin Model 8500, Nonin Medical Inc, Minnesota, USA). This sensor has a reported accuracy of ± 4 % between an SpO_2 ranging from 70-100% (Nonin Medical, Minnesota, USA). A second finger

clip pulse oximeter was used as an auxiliary measure (3100 WristOx, Nonin Medical Inc., Plymouth, USA). The wrist sensor has a reported accuracy of ± 2 digits (manufacturers guide). The earlobe was cleaned using an alcohol swab and the sensor attached so that the LED emitter and detector completely covered the earlobe. This method was preferred over fingertip measures as cycle ergometry was used throughout the trials reported in this thesis. Using the ear lobe as the sample site avoids the loss of signal when gripping the handlebars. The reproducibility and validity of the earlobe pulse oximeter was confirmed by Thake (2006) across a similar range of inspired fractional oxygen concentrations to that used in the current studies.

3.4.3 Indirect calorimetry

The Douglas bag technique was employed to measure ventilation, oxygen consumption, carbon dioxide production and respiratory exchange ratio (RER) during all preliminary incremental tests in order to prescribe absolute work intensities during each experimental chapter, as well as for respiratory measurements during each trial.

3.4.3.1 Calibration of gas analysers

The Servomex 1400 series gas analyser (Servomex, Crowborough, UK) was calibrated in the morning before testing and again immediately before any test samples. The calibration was performed at 3 points using medical grade gases (BOC, Guildford, Surrey) within an appropriate concentration range normoxia: O2 = 15, 18, 21% (room air) ; hypoxia: 8, 10, 12%); CO2; normoxia and hypoxia = 0, 0.04 (room air), and 5%. When calibrating the analyser the ‘zero’ gas (100% Nitrogen) was sampled for a minimum of 60 seconds and the display value then adjusted to the correct value using the zero button. The highest concentration was then used and

corrected after 60 seconds using the 'span' button. The mid-range gas was used to check the linearity of the system. During each testing session the mid-range gas was used to check for analyser drift. If this was detected the analyser was calibrated again before any measurement was made.

3.4.3.2 Measurement of gas volumes

Expired gas volumes were determined at an evacuation rate of $2L \cdot sec^{-1}$ via a dry gas meter (Harvard Dry Gas Meter, Cranlea, Birmingham, UK). The temperature of the expirate was determined via a digital thermocouple (Model 206-3722, RS Componants, Corby, UK) inserted into the sample outlet tubing of the dry gas meter. To check the accuracy of the dry gas meter a 3 L syringe was used to introduce air into the system at a rate of 20 strokes per minute. Barometric pressure was recorded using a digital barometer (TFA Wireless Weather Station, Cranlea, Birmingham, UK), and a whirling hygrometer (Brannan Thermometers. Cumbria, UK) used to measure wet and dry bulb temperatures for the determination of relative humidity.

3.4.3.3 Reliability of Douglas bag measurements

To assess the within day and between day reliability of the Douglas bag method, 8 males completed 2 exercise bouts, each separated by 5 days. Each exercise bout consisted of 10-minutes cycling at 100watts, and 10-minutes cycling at 150W. Douglas bag measurements were taken between minutes 6-7 and 8 – 9 of each work bout to assess within-trial reliability (4 observations per person, per trial), and also compared to the same measurements obtained during the second visit for each environmental condition. As no published data exists regarding acceptable error limits in gas exchange measurements (Atkinson et al., 2005). It was reasoned that

there would be no practical impact if the two measurements disagreed by more the 5L·min^{-1} ($\dot{V}_E$STPD), 0.2 L·min^{-1} ($\dot{V}O_2$ and $\dot{V}CO_2$) and 5ml·kg^{-1}·min^{-1} ($\dot{V}O_2$). Thus if the 95% limits of agreement fall within this region of equivalence then the systematic difference between the two measurements is likely not practically important. As a further measure for assessing homogeneity between measures, Cronbach's alpha was used. For clinical application of the variables measured, a minimum alpha level of 0.9 is required, with 0.95 being desirable (Bland and Altman, 1997). Test retest data is presented in Table 3.4.1 and mean differences in Figure 3.4.1.

Table 3.4.1. Test – retest gas exchange variables (mean ± SD, *n* = 8) measured by the mixing box and Douglas bags during submaximal normoxic and hypoxic exercise.

Gas exchange variables	Test one mean (SD)	Test two mean (SD)	P value	Difference %	Cronbach's Alpha
Douglas Bag					
$\dot{V}O_2$ (L·min^{-1})	1.9 ± 0.45	1.88 ± 0.44	.006	1.06	.994
$\dot{V}O_2$ (ml.kg^{-1}·min^{-1})	26.3 ± 7.4	26.4 ± 7.7	.876	0.15	.983
$\dot{V}CO_2$ (L·min^{-1})	1.74 ± 0.45	1.68 ± 0.45	<0.01	3.57	.995
$\dot{V}_E$ (L·min^{-1})	38.9 ± 1.5	39.3 ± 1.9	.482	.84	.956

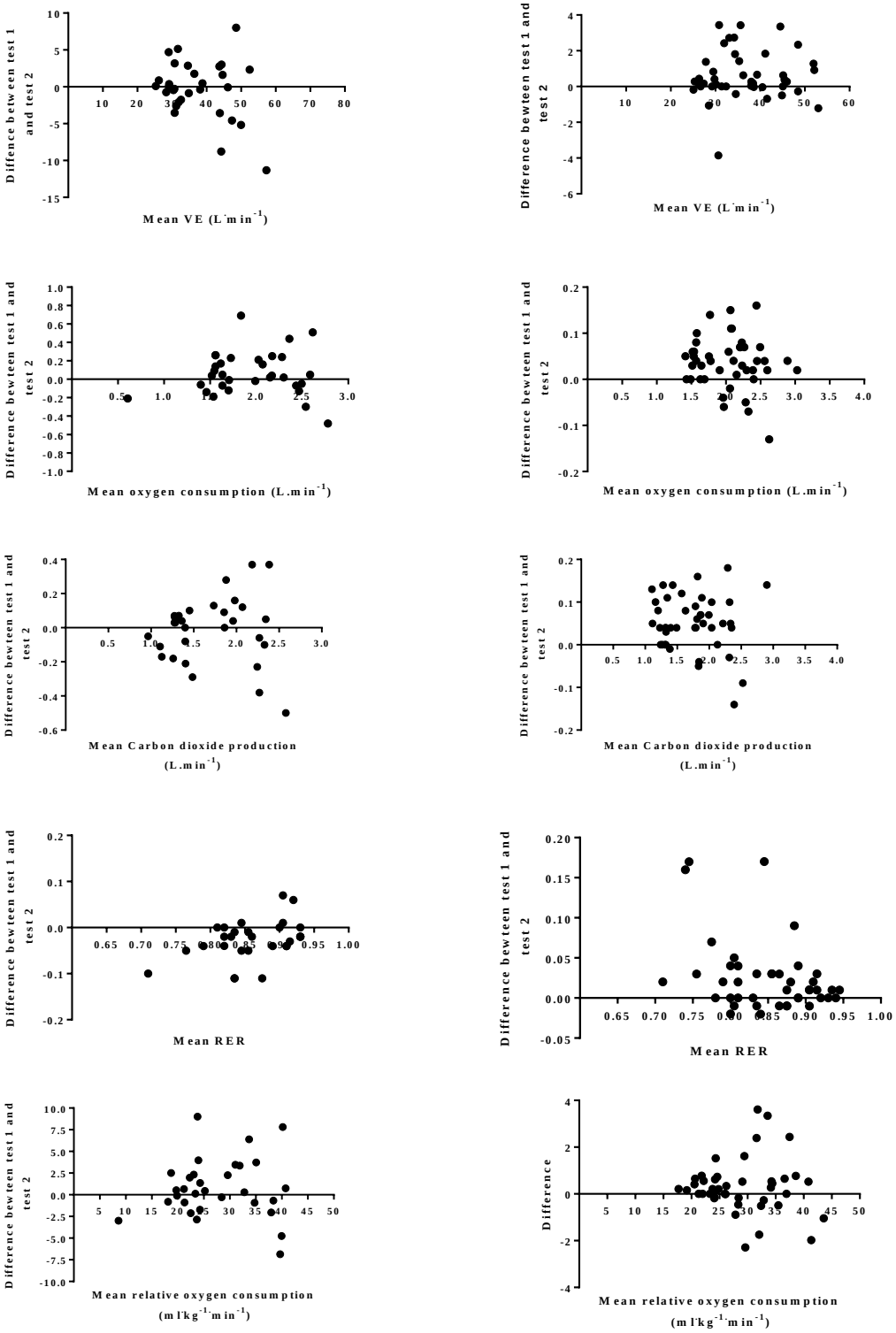

Figure 3.4.1. Tukey difference plots between test 1 and test 2 for respiratory measurements. The mean differences were 0.42L·min^{-1} (3.48 to – 2.63) for ventilation, 0.02L·min^{-1} (0.16 to – 0.11) for oxygen consumption, 0.05L·min^{-1} (0.18 to

– 0.07 for carbon dioxide production and 0.13ml·kg^{-1}.min^{-1} (2.69 to – 2.43) for relative oxygen consumption.

The differences between test 1 and test 2 were within the accepted limits discussed by Atkinson et al., (2005) and recommended by BASES, if properly calibrated equipment is employed and biological variability accounted for. All variables for both displayed alpha values above 0.95. It can therefore be concluded that the Douglas bag system used is a valid and reliable tool in the assessment of gas exchange variables during submaximal exercise.

3.4.4 Subjective measures

Participants were asked to provide a rating of perceived exertion (RPE) (Borg, 1970) and thermal sensation (TS) (Toner et al., 1986) at regular intervals throughout each trial. Participants reported RPE in whole numbers that ranged from 6 (very, very light) through 13 (somewhat hard) to 20 (very, very hard) (Borg, 1970). The TS scale was reported in 0.5 units and ranged from 0.0 (unbearably cold), through 4.0 (comfortable), to 8.0 (Unbearably hot) (Toner et al., 1986).

3.4.5 The Acute Mountain Sickness Questionnaire (AMS)

A version of the self-assessment section of the Lake Louise Acute Mountain Sickness questionnaire (Roach et al., 1996), modified and used by Thake (2006) was used throughout each study to assess the well-being of each subject. This scale was completed at defined intervals throughout each trial. Details of the sample times can be found in each experimental chapter.

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3.5 Haematological measurements

3.5.1 Blood lactate

Blood lactate and blood glucose were sampled from a fingertip capillary using a calibrated Biosen C-line analyser (EKF Diagnostics, Magedburg, Germany). The analyser was calibrated prior to each use using known standards within the physiological range (5mmol, 12mmol). Prior to sampling the fingertip was prepared with an alcohol swab and allowed to dry before lancing. The skin was punctured using an automated safety lancet (Sarstedt Saftey Lancet, Sarstedt, Numbrecht, Germany). The first drop of blood was wiped away using tissue and a free flowing sample collected into a capillary tube (~ 25µl). Details of timing of collection can be found in each experimental chapter.

3.5.2 Plasma Volume changes

Hematocrit (Hct) and hemoglobin [Hb] were measured throughout each trial in order to determine any change in plasma volume. Specific details of time points used in each study are stated in each of the experimental chapters. Haematocrit was analysed in triplicate by collecting 100µL of whole blood in heparinised capillary tubes from a fingertip puncture warmed via water to ensure arterialised blood. Filled capillary tubes were spun at 5000rpm for 15 minutes in a micro Hct centrifuge, with Hct values quantified using a micro Hct reader (Hawksly and Sons, Surrey, UK). Hemoglobin was measured using whole blood collected in triplicate from the same measurement site as Hct. A 10µL sample was collected into a cuvette and analysed for [Hb] using a calibrated Hemocue device (Hemocue, Sheffield, UK). The Hemocue was calibrated prior to each use by inserting a calibration curvette into the analyser and insuring the

result was within the manufactures instructions. For measurement a mean value was collected from the 3 samples and if there was a difference of more than 0.3g•dL^{-1} between samples the test was repeated (Rechner et al., 2002). Plasma volume changes were calculated using the equations of Dill and Costill (1974; Equation 3.1). Coefficents of variation (CV) were determined from 20 blood samples analysed in duplicate and were 2.1% for Hct and 2.8% for Hb respectively.

Equation 3.1:

$$\%\Delta PV = [(Hb_0/Hb_1) \cdot (1-Hct_1/Hct_0)] \cdot 100$$

%ΔPV = percentage change in plasma volume

Hb_0 = Hb concentration at baseline

Hb_1 = Hb concentration at a given time point

Hct_1 = Hct at a given time point

Hct_0 = Hct concentration at baseline.

3.6.1 Measurement of lactate threshold and $\dot{V}O_{2\ peak}$

The lactate threshold and $\dot{V}O_2$ peak tests were used to assess each participant's aerobic fitness to establish the resistance required to elicit the desired exercise intensity during the main experimental trials in each experimental chapter. Starting workload was set at a calculated power output of 70 Watts (W) with a target cadence of 70 rev•min^{-1}. The workload was increased by 35W every 4 minutes until a blood lactate value above 4mmol•L^{-1} was reached. Thereafter workload was increased by 35W every two minutes until volitional exhaustion. Peak power output was defined as the highest workload sustained for 1 minute (Bentley et al., 2001). Fingertip whole blood samples were collected into sodium heparinised capillary tubes (20µL; EKF

Diagnostics, Magdeburg, Germany) and stored in 1mL Eppendorf tubes during the last 30 seconds of each 4-minute stage. Samples were analysed for blood lactate using a Biosen C-Line analyser (EKF Diagnostics, Magdeburg, Germany). Oxygen uptake was recorded using open circuit spirometry with expired gas collected into 200L Douglas bags (Cranlea and Company, Birmingham, UK) for approximately 45 seconds during the last minute of each stage during the LT test and during every second minute thereafter, as was heart rate (Suunto, T6c, Vantaa, Finland). Expired gas was analysed using a rapidly responding Servomex paramagnetic analyser series 1440 for oxygen and an infrared carbon dioxide analyser (Servomex, Crowborough, UK). To determine gas volumes, a Harvard Dry Gas meter (Cranlea, UK, Birmingham) was used in conjunction with a vacuum pump to evacuate the Douglas bags at a rate of $2L \cdot sec^{-1}$. The temperature of the expirate was determined via a thermocouple (Thermocouple 206-3722; RS Components, Corby, UK) sited within the outlet tubing of the dry gas meter.

3.6.2 Reliability of measuring peak oxygen uptake in normoxia and hypoxia

To assess the reliability and reproducibility of the protocol described in Section 3.6.1, ten participants completed two sea level lactate threshold and $\dot{V}O_{2\ peak}$ tests, separated by at least one week. Six of these participants also completed the protocol when breathing normobaric hypoxia ($F_IO_2 = 0.14$). Repeat tests were conducted at the same time of day. Differences between test-retest physiological responses were analysed using the coefficient of variation (CV), expressed as the group standard deviation divided by the grouped mean values, (Table 3.6.1). Test-retest variability was expressed as the percentage difference between tests (Bland and Altman, 1986; Figure

3.6.2, 3.6.4). The typical error of measurement for peak power output, oxygen consumption, carbon dioxide production, RER and minute ventilation (both STPD and BTPS) was also calculated by dividing the standard deviation of the difference score between trials by √2 (Hopkins, 2000).

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Table 3.6.1 Mean ± SD peak physiological and respiratory responses to the normoxic lactate threshold and $\dot{V}O_{2\ peak}$ test in normoxia (N, *n* = 8) and hypoxia (F_IO_2 = 0.14, equivalent to 3000m, HYP, *n* = 6). C.V = coefficient of variation.

	Normoxia			Hypoxia		
	Test 1	Test 2	C.V	Test 1	Test 2	C.V (%)
Peak Power (Watts)	280 ± 33	282 ± 27	0.43	217 ± 41	220 ± 39	2.8
Heart rate (beats•min^{-1})	186 ± 7	186 ± 6	0.15	187 ± 4	187 ± 2	0.4
SpO_2 (%)	95 ± 3	95 ± 4	2.4	78 ± 4	79 ± 4	3.6
Peak BLa (mM)	10.7 ± 1.2	11.8 ± 1.6	7.1	11.4 ± 2.2	11.3 ± 1.7	0.8
$\dot{V}O_2$ peak (L•min^{-1})	3.40 ± 0.36	3.43 ± 0.38	0.5	2.40 ± 0.25	2.42 ± 0.24	0.7
$\dot{V}CO_2$ peak (L•min^{-1})	3.81 ± 0.45	4.12 ± 0.54	5.5	2.81 ± 0.53	2.85 ± 0.50	1.2
RER	1.12 ± 0.07	1.20 ± 0.10	4.9	1.17 ± 0.13	1.18 ± 0.14	0.6
$\dot{V}_E$ (BTPS) (L•min^{-1})	120 ± 15.5	126 ± 18	3.3	96 ± 18	99 ± 19	1.8
$\dot{V}_E$ (BTPS)/ $\dot{V}O_2$ (STPD)	35.4 ± 4.1	36.75 ± 4.8	2.6	39.9 ± 3.7	40.5 ± 5.8	1.2
$\dot{V}_E$ (BTPS)/ $\dot{V}CO_2$ (STPD)	31.6 ± 2.9	30.6 ± 3.4	2.3	34.8 ± 9.9	34.5 ± 2.2	0.8
RPE	19 ± 1	19 ± 1	0.6	18 ± 1	19 ± 1	0.7

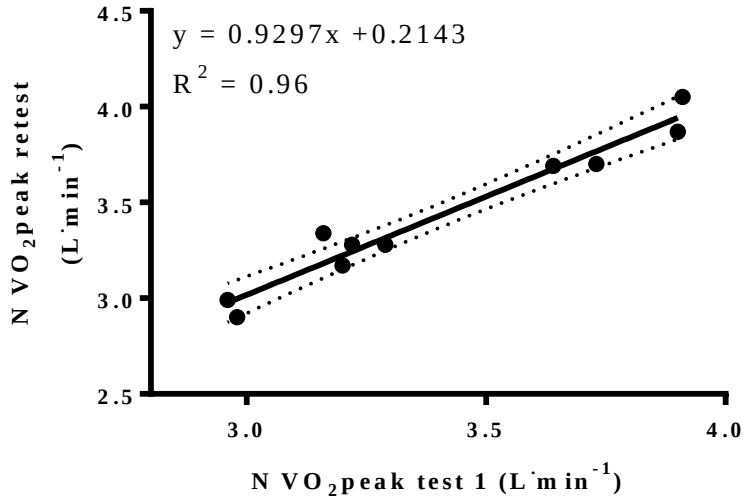

Figure 3.6.1. The relationship between normoxic test and retest (n = 10). $\dot{V}O_{2\ peak}$ data, r = 0.976. Each data point represents an individual participant.

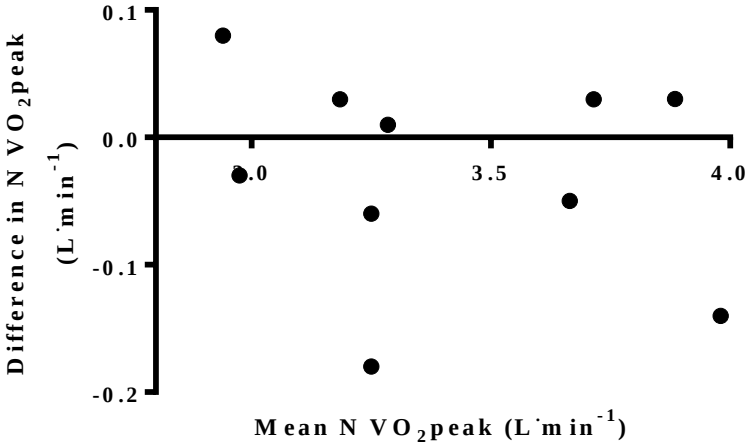

Figure 3.6.2. Tukey difference plot for the difference between normoxic VO_{2peak} values plotted against the mean normoxic $\dot{V}O_{2\ peak}$ values for each subject (n = 10).

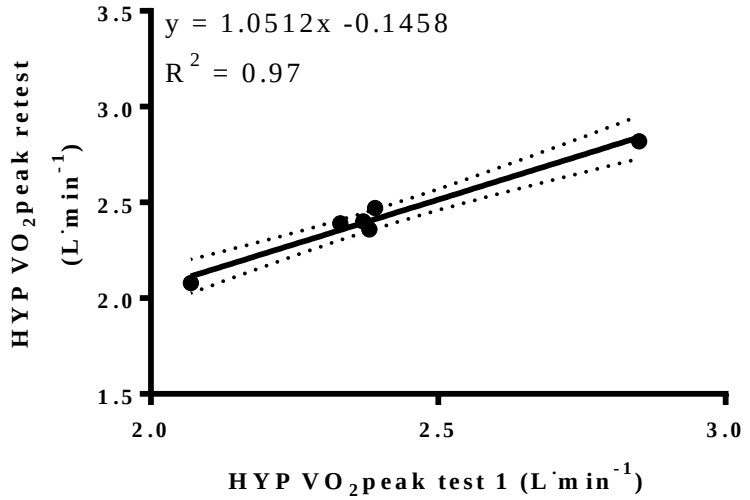

Figure 3.6.3. The relationship between hypoxic test and retest (*n* = 6). $\dot{V}O_{2\ peak}$ data, r = 0.987. Each data point represents an individual participant.

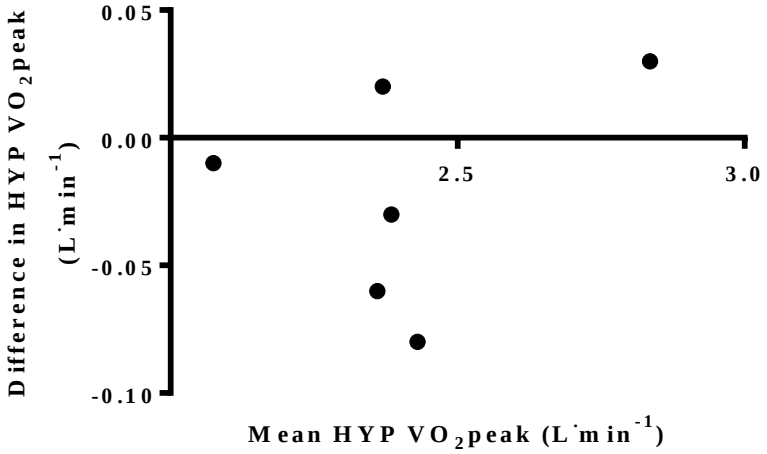

Figure 3.6.4. Tukey difference plot for the difference between hypoxic $\dot{V}O_{2\ peak}$ values plotted against the mean hypoxic $\dot{V}O_2$ peak values for each subject (*n* = 6).

When measuring peak $\dot{V}O_2$ it is accepted that measurement error may result from biological variation, measurement precision, and random error. Sandels (2003) determined the overall precision of $\dot{V}O_2$ determination to be 1.4%, and that the level of precision increased as exercise intensity increases for a given collection period, or if the expirate collection duration increases for a given exercise intensity. During severe exercise, a Douglas bag collection made over a 45 second period could be expected to yield a precision of 0.9% (Sandels, 2003). Using the described protocol for $\dot{V}O_2$ peak, the technical error of measurement (TEM) was 0.81 ± 2.46 and 0.98 ± 1.74% in N and HYP respectively. The between test $\dot{V}O_2$ values also showed highly significant correlations in both N (0.976) and HYP (0.987) and low C.V. (N = 0.5, HYP = 0.7). It can be concluded that peak physiological responses can be reliably determined using the cycle ergometry protocol, in both normoxic and hypoxic conditions, in this thesis.

3.7 Thermoregulatory measurements

Rectal temperature was chosen as the criterion core temperature measurement site from a depth of 10cm past the anal sphincter. Skin temperature (T_{skin}; Squirrel Meter Logger, Grant Instruments, Cambridge, UK) was recorded continuously throughout each trial using telemetry (Eltek Limited Cambridge, UK), to an accuracy of ± 0.1°C from the right side of the body when stood in the anatomical reference position, at the belly of the following muscles: pectoralis major; lateral head of the triceps brachii; rectus femoris; and the lateral head of the gastrocnemius. Mean skin temperature was calculated using equation 3.2 (Ramanathan, 1964).

Equation 3.2. Calculation of mean skin temperature

$$T_{sk} = 0.3\ (t_{chest} + t_{arm}) + 0.2\ (t_{thigh} + t_{calf})$$

The accuracy of the temperature probes was assessed by submersing them in a water bath at 30°C. The water bath was then programmed to heat up to 40°C. Measurements from the probes were taken every 2 minutes, until 40°C was reached, and compared to a mercury laboratory thermometer.

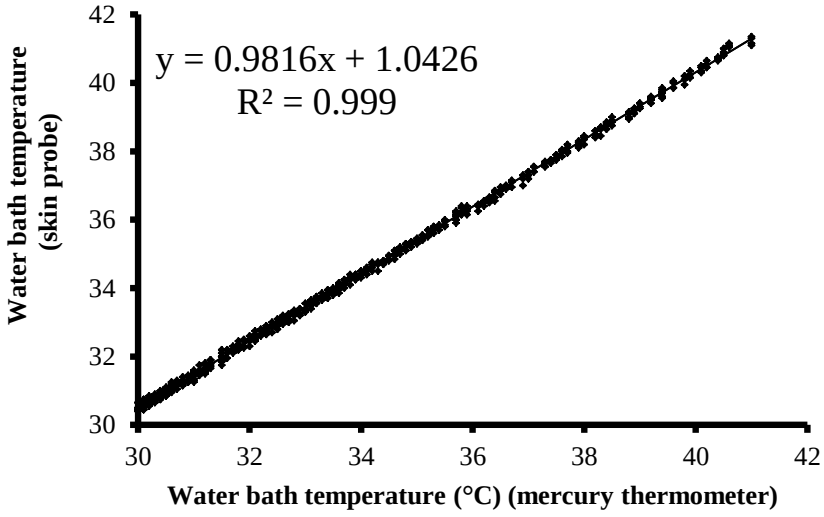

Figure 3.7.1 Water bath temperature (°C) measured from a mercury thermometer and skin temperature probes, over a dynamic temperature range (30-40°C). Skin temperature probes that differed by more than 0.2°C were not used for testing. Data represent individual data points for 4 skin temperature thermistors and 1 rectal probe. When new probes were required they were assessed in this way.

3.7.1. Sweat rate calculation

Sweat losses were determined by the change in nude body mass pre to post exercise. Sweat volume and mass were considered equal (i.e. 1 mL = 1 g) and were expressed as a rate (volume per unit time). Participants weighed themselves nude before and within 5 minutes

following the completion of exercise to the nearest 0.1kg (Seca, model 875, Seca, Birmingham, UK).

3.8.1 Calculation of physiological strain

The physiological strain index (Moran et al., 1998) is a simple method for assessing heat strain, and ranges from 0 (no to little strain) to 10 (very high strain; Table 3.8.1) using Equation 3.3.

Equation 3.3. Calculation of the physiological strain index (PSI)

$$PSI = 5(T_{core-t} - T_{core0}) \cdot (39.5-T_{core0})^{-1} + 5(HR_t - HR_0) \cdot (180-HR_0)^{-1}$$

Where core temperature (T_{core0} and HR_0) are the initial T_{core} and HR, respectively and where T_{core-t} and HR_t are simultaneous measurements taken at any time (Moran et al., 1998). The PSI has been shown to be sensitive enough to differentiate between levels of heat strain induced by different clothing ensembles and types of heat stress (hot, dry versus hot, wet; Moran et al., 1998b), hydration status (Moran et al., 1998a), rest and exercise periods during intermittent walking (Gotshall et al., 2001), cooling during exercise (Chevront et al., 2003) and different age groups and genders during heat acclimation (Moran et al., 2002).

3.9 Heat and hypoxic acclimation

In Chapters 5 and 6 a period of acclimation, to either heat or hypoxia, was conducted. Details of the number of repeated daily exposures can be found in the respective experimental chapters. All acclimation sessions consisted of a 15-minute stabilisation period for resting measurements to be obtained, followed by a 15 minute seated and resting "wash in" period and 60 minutes of cycling at 50% normoxic $\dot{V}O_2$ peak. Measurements of heart rate, core temperature, SpO_2, RPE, TS, and PSI were taken at the end of rest and throughout the

exercise period. All heat sessions were conducted at ~40°C, 20% RH, and all hypoxic acclimation sessions were performed at a simulated altitude of 3095m asl, F_IO_2 ~0.14; ~18°C, 30-40%RH as described in Section 3.3.1. Control groups performed the same level of exercise at sea level in an air condition laboratory (~18°C, 30-40%RH). Details of experiment specific measurements and time points during the rest and exercise period can be found in the respective experimental chapters.

Table 3.8.1. Evaluation and categorisation of different levels of heat strain by the physiological strain index (PSI) (Moran et al., 1998).

3.10 Determination of the workload to be used in study 1

The general aim of experiment 1 (Chapter 4) was to compare the physiological and cellular responses to heat, hypoxia and heat and hypoxia together, at rest and during submaximal exercise. Furthermore the short term preconditioning effect of a prior exposure to these

stressors, and their role on heat stress proteins and other markers of cellular protection, was to be investigated.

Exercise above the anaerobic threshold has been shown to result in different stress hormone responses (Ortega et al., 2010), increased levels of oxidative stress (Quindry et al., 2003), an over-proportional excretion of stress hormones and immunological markers (Gabriel et al., 1997) and altered heat stress protein dynamics (Ogawa & Fehrenbach, 2010).

Thus to enable direct comparisons between environmental stressors it was important to confirm that oxygen consumption during exercise corresponding to 50% normoxic $\dot{V}O_2$ peak remained below anaerobic threshold. In this instance the anaerobic threshold was defined using the D_{max} method described by Cheng et al., (1992). This method determines the maximal perpendicular distance of the lactate curve from the line connecting the start with the endpoint of the lactate curve. This method was chosen to locate the anaerobic threshold as it is objective and can be determined in all participants. All blood lactate markers were calculated using the free statistics package R (www.cran.r-project.org) and freely available code purposely written for the determination of lactate threshold markers (Lactate-R, Newell et al., 2007)

The workload of 50% normoxic $\dot{V}O_2$ peak was selected, for this study, as prior research had shown this workload to be below the anaerobic threshold when subjects were exercising in a similar level of hypoxia to be used in the present series of investigations, allowing comparisons to be made between both relative and absolute workloads in normoxia and hypoxia (Thake, 2006). Previous research has also shown that this workload allowed subjects to remain below the anaerobic threshold during similar activity performed in acute heat stress (Castle et al., 2006, McClung et al., 2008) similar to the heat stress to be employed in the current series of investigations. As these studies exposed subjects to both heat and hypoxia

together it was important to undertake preliminary studies to ensure that 50% normoxic $\dot{V}O_2$ peak was sub-threshold in the combined stress of heat and hypoxia. This was necessary as no previous study has investigated the combination of heat and hypoxia within a laboratory environment. Sub-threshold in this instance was defined as an oxygen consumption value below that obtained at the anaerobic threshold, and generating stable blood lactate values through-out the protocol.

Eight males (age, 22.1 ± 5.3years; body mass, 76.8 ± 10.9kg; height, 1.76 ± 0.06m) completed lactate threshold and $\dot{V}O_{2\ peak}$ test in both normoxia and hypoxia ($F_IO_2 = 0.14$) in a randomized, counterbalanced order, separated by at least 7 days. The aerobic lactate threshold (defined as a 1mmol increase from resting BLa values), and anaerobic threshold assessed via the D_{max} method were assessed for each participant. Between test data was analysed using a paired t-test. Within group differences between thresholds were examined using a one-way ANOVA. The significance level was set at $P<0.05$.

3.10.1 Maximal and submaximal physiological responses

Values attained at the end of each maximal exercise test are reported in Table 3.10.1 Peak power, $\dot{V}O_2$, $\dot{V}CO_2$, SpO_2, were lower in hypoxia ($P <0.05$), whereas RER, and ventilatory equivalents for $\dot{V}O_2$, $\dot{V}CO_2$ were greater in hypoxia. No differences were observed in peak blood lactate and RPE. Hypoxic $\dot{V}O_{2\ peak}$ was attained at 77 ± 13 of normoxic $\dot{V}O_{2\ peak}$ (range 62-89%). Participants reached their normoxic aerobic lactate threshold at 59 ± 9 % of their normoxic $\dot{V}O_{2\ peak}$. In hypoxia participants reached their aerobic lactate threshold at 58 ± 15% of hypoxic $\dot{V}O_{2\ peak}$. This corresponds to 46 ± 12 % (range 33 – 72%) of normoxic $\dot{V}O_{2\ peak}$. This is lower than previously reported (Thake, 2006; 63 ± 5 %). This is likely due to the higher levels of aerobic fitness displayed in the subjects used in previous research (4.26

± 0.46L•min^{-1}, Thake, 2006, versus 3.73 ± 0.7L•min^{-1} in this current investigation). The hypoxic BLa threshold occurred at 1.66 ± 0.44 L•min^{-1} and was lower than the oxygen consumption at 50% normoxic $\dot{V}O_2$ peak (1.81 ± 0.23L•min^{-1}). It can be concluded that the workload of 50% $\dot{V}O_2$ peak corresponds to a workload slightly above the hypoxic aerobic threshold, as defined as a 1mmol•L^{-1} increase in blood lactate from rest and below the anaerobic threshold as determined by the Dmax method. Therefore, the workload of 50% $\dot{V}O_2$ peak is appropriate for use in Chapter 4 using a participant population with similar physical characteristics.

Table 3.10.1. Mean ± SD peak physiological responses to a maximal exercise test in both normoxia (N) and hypoxia (H) (n = 8).

	PPO (Watts)	$\dot{V}_E$ **BTPS (L•min^{-1})**	$\dot{V}_E$ **STPD (L•min^{-1})**	$\dot{V}O_2$ **(L•min^{-1})**	$\dot{V}CO_2$ **(L•min^{-1})**	**RER**	**HR (Beats•min^{-1})**	**SPO$_2$ (%)**	**BLa (mmol•L^{-1})**	**RPE**
N	264 ± 58	121 ± 18.2	100 ± 14.5	3.62 ± 0.46	3.92 ± 0.64	1.08 ± 0.09	182 ± 11	96 ± 2	11.5 ± 2.96	20 ± 0.00
H	229 ± 45	122 ± 23.5	101 ± 19.2	2.99 ± 0.66	3.44 ± 0.70	1.16 ± 0.14	181 ± 14	81 ± 3	10.3 ± 2.14	20 ± 0.00

3.10.2 Anaerobic thresholds

The $\dot{V}O_2$ at each threshold was greater in normoxic conditions compared to hypoxia ($P < 0.001$). The normoxic anaerobic threshold, defined by Dmax, occurred at 72 ± 8 % N $\dot{V}O_2$ max, and a similar threshold intensity was seen in hypoxia (77 ± 8% of H $\dot{V}O_2$).

Table 3.10.2. Mean ± SD oxygen consumption, heart rate, and power output at lactate threshold, 4mmol.L^{-1} lactate, and D-max during normoxia and hypoxia (*n* = 8).

	Lactate threshold	**4 mmol^{-1}**	**D_{max}**
$\dot{V}O_2$ (L•min^{-1})			
Normoxia	2.12 ± 0.39	2.55 ± 0.36	2.61 ± 0.43
Hypoxia	1.66 ± 0.44	2.06 ± 0.49	2.35 ± 0.44
HR (beats•min^{-1})			
Normoxia	140 ± 20	159 ± 18	159 ± 13
Hypoxia	138 ± 20	158 ± 21	163 ± 12
Power Output (W)			
Normoxia	152 ± 60	197 ± 70	196 ± 51
Hypoxia	138 ± 53	173 ± 66	183 ± 51

The workload of 50% normoxic $\dot{V}O_2$ max (1.81L.min^{-1}) corresponds to 62 ± 8 % of the hypoxic $\dot{V}O_2$ max and was below the normoxic and hypoxic anaerobic threshold (Dmax) in each participant.

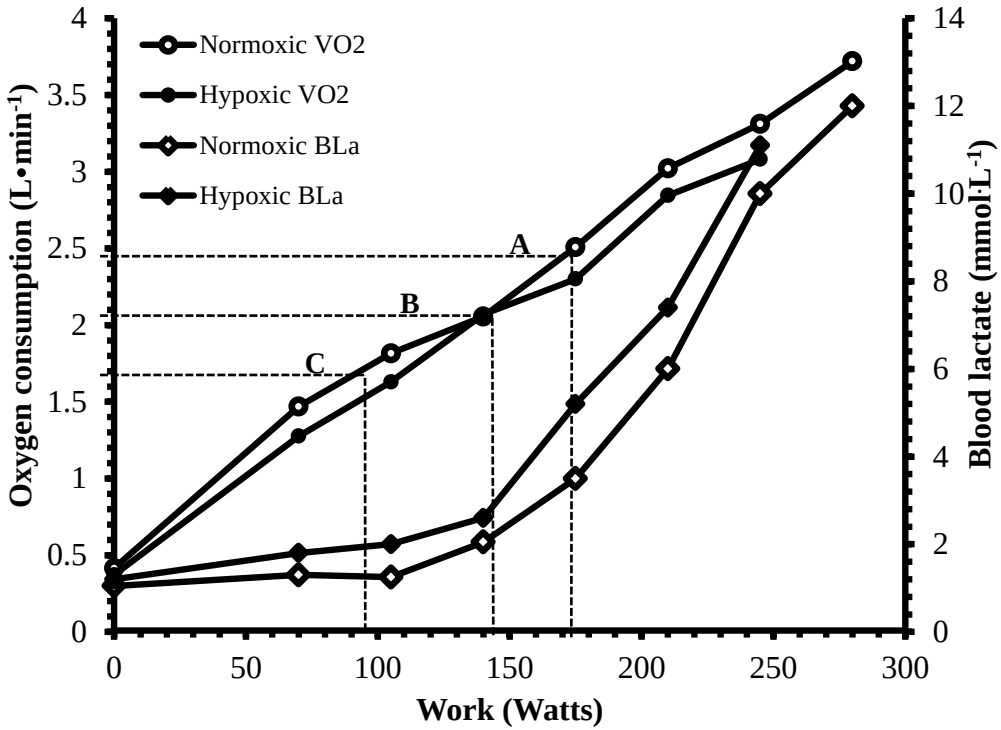

Figure 3.10.1. Oxygen consumption and blood lactate responses to the incremental exercise test in normoxia and hypoxia in a representative participant. A = Normoxic D_{max}, B = Hypoxic D_{max}, C = 50 % Normoxic $\dot{V}O_{2\ peak}$.

3.10.3 Physiological responses to acute exposure to 40˚C heat and moderate hypoxia.

As there is little literature regarding acute exposure to both heat and hypoxia at the same time, a further preliminary study was needed to determine that participants were exercising below their sea level and hypoxic anaerobic threshold across all trials. To enable direct comparisons between the environmental conditions used within this thesis it was important to confirm that oxygen consumption during exercise corresponding to 50% normoxic $\dot{V}O_2$ peak remained below ones anaerobic threshold, assessed herein from the Dmax method described in Section 3.6.1.

Participants completed a constant load exercise bout in the conditions to be used in study 1 (Chapter 4). Upon arrival at the laboratory participants inserted a rectal thermistor (Grant instruments, Cambridge, UK) 10cm past the anal sphincter and were fitted with a heart rate monitor (Suunto T6c, Finland), and skin temperature thermistors (Grant Instruments, Cambridge, UK) attached to the calf, thigh, upper arm and chest for determination of mean skin temperature (Equation 3.2). Following a ten-minute resting period fingertip capillary samples were drawn in triplicate for the assessment of whole blood lactate (Biosen C-Line analyser, EKF Diagnostics, Germany). Hemoglobin and haematocrit were determined as outlined in Section 3.8.2.

Participants then entered the environmental chamber and commenced a 30-minute resting period, allowing for the wash-in of the hypoxic gas. The hypoxic gas was delivered via 1000L Douglas bags filled using an oxygen filtration device (Hypoxico HYP123 Hypoxicator, New York, USA) as described in Section 3.3.1. Following rest participants commenced cycling at 50% of their assessed normoxic $\dot{V}O_{2\ peak}$, as determined via regression

analysis, for 90 minutes at a cadence of 70rev•min^{-1}. Measures of $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}_{E\ STPD}$, and RER were collected every 15 minutes via the Douglas bags. Immediately after Douglas bag collections fingertip capillary samples were collected in triplicate for assessment of blood lactate, Hb, and Hct as described in section 3.8.2. Subjective measures of perceived exertion (The Borg Scale, Borg, 1970), Thermal sensation (Moran et al., 1996) and Acute mountain sickness (the adapted Lake Louise questionnaire) were also collected every 15 minutes throughout the protocol.

3.10.4 Respiratory and cardiovascular responses to acute heat and hypoxia

Only 2 of the 8 participants were able to complete the full 90-minute cycling protocol (61 ± 24 min, range 30 – 90 min). During these environmental conditions the mean ± SD final oxygen consumption was 1.79 ± 0.62 L•min^{-1}, which is below the calculated hypoxic lactate threshold value of 2.35 ± 0.51 L•min^{-1}. This indicates that the work rate of 50% N $\dot{V}O_{2\ peak}$ allows participants to work below both sea level and hypoxic lactate threshold across trials of differing environmental stress throughout a significant portion of the exercise period. It is likely that significant cardiac drift contributed to higher oxygen consumption values when participants were entering the final stages of this combined environmental stressor, and perceptual sensations lead to the early termination of trials in participants that were working well below maximal heart rate (e.g. participants 6 and 7).

Table 3.10.3. Physiological responses to the acute exposure to heat and hypoxia upon either completion of 90 minutes exercise or voluntary exhaustion. $\dot{V}O_2$ values in bold correspond to those over the hypoxic lactate threshold.

Participant	Time to exhaustion	Heart rate (beats•min^{-1})	Rectal temperature (°C)	$\dot{V}_E$ BTPS (L•min^{-1})	$\dot{V}_E$ STPD (L•min^{-1})	$\dot{V}O_2$ (L•min^{-1})	$\dot{V}CO_2$ (L•min^{-1})	RER	BLa (mmol•L^{-1})	PSI (A.U)
1	45	154	37.90	51.6	42.7	1.26	1.32	1.05	3.28	5.67
2	62	175	38.40	66.4	54.7	1.74	1.84	1.06	2.30	7.12
3	73	182	39.25	66.8	73.7	**3.10**	2.55	0.82	3.45	9.52
4	90	174	38.45	58.0	47.5	1.82	1.69	0.93	2.30	7.66
5	90	165	38.20	45.0	35.9	1.26	1.24	0.99	2.10	6.69
6	30	163	38.15	57.9	47.8	**2.28**	1.79	0.78	5.66	5.94
7	30	154	38.45	56.8	46.8	1.43	1.22	0.85	2.55	3.39
8	71	188	38.40	54.0	44.6	1.45	1.07	0.74	3.41	5.17
Mean	61	169	38.40	57.1	49.20	1.79	1.59	0.90	3.13	6.40
SD	24	12	0.40	7.20	11.20	0.62	0.48	0.12	1.15	1.80

There is substantial inter-individual variation in both the response to this novel exercise challenge, and the ability to tolerate such a challenging protocol. The decision was made to utilise this workload (50% $\dot{V}O_2$ peak) in experiment 1 (Chapter 4) in order to further examine the physiological and biochemical responses to a combined environmental stressor, and to then compare these absolute responses with heat and hypoxia in isolation.

3.11 Reliability and sensitivity of a hypoxic stress test

In each experimental chapter, the physiological responses to fixed load work in moderate hypoxia (F_IO_2 0.14) are important. To determine the reliability and sensitivity of the hypoxic stress test described below, 6 males (height, 1.77 ± 0.05m; mass, 74.7 ± 6.9kg; normoxic $\dot{V}O_{2\ peak}$, 3.50 ± 0.67L•min^{-1}) completed 5 experimental trials, each separated by at least 7 days. The first two experimental sessions consisted of a lactate threshold and $\dot{V}O_{2\ peak}$ test, as described in Section 3.6.1, in either normoxic, or hypoxic conditions in a counterbalanced fashion. Participants then returned to the laboratory on 3 occasions to complete an identical bout of work under moderate hypoxia in order to determine how many familiarisation sessions would be required prior to collecting experimental data for each chapter.

Upon arrival to the laboratory participants voided their bladder to provide a sample for assessment of urine specific gravity (USG; Visual refractometer, Index Instruments, Cambridge, UK) and osmolality (Osmocheck, Vitech Scientific, Partridge Green, West Sussex, UK). Participants were considered euhydrated if these values were <1.030 and 600m mOsmol•kg^{-1} respectively (Yamada et al., 2008; Armstrong 1994). Participants then measured their own nude body mass (Seca 899 scales, Seca, Hamberg, Germany) and attached a heart rate monitor to their chest (Suunto T6c, Suunto, Vaanta, Finland). Participants were seated on a cycle ergometer (Monark Ergomedic 874e, Vandsbro, Sweden)

and completed a 15-minute resting period breathing hypoxic gas delivered via a series of 1000L Douglas bags (Section 3.3.1). Participants completed 60 minutes of cycle exercise at an intensity corresponding to 50% normoxic $\dot{V}O_{2\ peak}$. Measures of heart rate, arterial oxygen saturation (S_PO_2), and respiratory measures via Douglas bags were taken at the start and end of the 15-minute rest period, and every 10 minutes throughout exercise. Resting respiratory Douglas bag collections lasted 5 minutes to ensure a sufficient volume of expired gas was in the bag. Exercising measurements were approximately 60 seconds. Fingertip capillary samples were collected before and after rest, and every 10 minutes throughout for the assessment of blood glucose and blood lactate using a Biosen analyzer (Biosen C-Line, EKF Diagnostic, Microberg, Germany). Heparinized capillary sample tubes were collected in triplicate and used to establish hematocrit and hemoglobin as described in Section 3.8.2.

The mean exercising physiological responses during each HST are given in Table 3.11.1. A 3 (trial) x 7 (time) repeated measures ANOVA found no significant differences within any of the physiological variables measured between any of the HST. The typical error of measurement and coefficient of variation for mean physiological measurements collected during exercise for each test are shown in Table 3. 11. 2. Based on the small typical error and low CV values observed for the physiological variables measured within HSTs it was reasoned that a familiarisation session prior to experimental hypoxic stress tests would not be required during subsequent studies. This decision was taken to reduce the time commitment placed on participants and to reduce the potential adaptive responses that may occur in some individuals to repeated brief hypoxic exposures.

Table 3.11.1. Exercise physiological responses during each hypoxic stress test (*n* = 6; Mean ± standard deviation).

HST	Heart rate (beats•min^{-1})	SpO_2 (%)	$\dot{V}_E$ BTPS (L•min^{-1})	$\dot{V}_E$ STPD (L•min^{-1})	$\dot{V}O_2$ (L•min^{-1})	$\dot{V}CO_2$ (L•min^{-1})	RER	BLa mmol•L^{-1}
1	148 ± 13	79 ± 3	60.7 ± 7.0	49.4 ± 5.9	1.96 ± 0.2	1.89 ± 0.2	0.97 ± 0.07	3.90 ± 1.35
2	148 ± 14	79 ± 3	62.4 ± 5.0	50.6 ± 4.6	1.99 ± 0.2	1.97 ± 0.1	1.00 ± 0.11	3.67 ± 1.40
3	148 ± 14	80 ± 3	63.5 ± 3.1	51.5 ± 3.1	2.02 ± 0.2	1.97 ± 0.1	0.98 ± 0.08	3.56 ± 1.25

Table 3.11.2. Technical error of measurement and coefficient of variation for selected exercising physiological variables during repeated hypoxic stress tests separated by 7 days (n = 6).

	Technical error of measurement			Co-efficient of variation (%)		
Variable	HST1 vs HST2	HST 1 vs HST3	HST2 vs HST 3	HST1 vs HST2	HST 1 vs HST3	HST2 vs HST 3
HR (beats•min^{-1})	1.6	2.6	1.7	0.9	1.6	0.9
SpO_2 (%)	0.9	1.1	0.5	0.8	1.3	0.6
$\dot{V}_E$ BTPS (L•min^{-1})	3.92	4.85	2.35	4.8	6.5	2.9
$\dot{V}_E$ STPD (L•min^{-1})	2.81	1.99	2.93	4.2	6.3	2.7
$\dot{V}O_2$ (L•min^{-1})	0.11	0.17	0.16	4.0	6.0	6.0
$\dot{V}CO_2$ (L•min^{-1})	0.12	0.16	0.08	6.0	7.0	2.0
RER	0.05	0.06	0.10	4.0	4.0	6.0
BLa (mmol•L^{-1})	0.62	0.52	0.39	15.0	14.0	8.7

3.11.1 Reliability and sensitivity of a combined tolerance test and cycling performance test in both normoxia and hypoxia.

For the final study (Chapter 6) the aim was to assess both tolerance and performance in hypoxia. To determine the biological and technical variation of the hypoxic tolerance and performance test (HTPT), and to assess whether any difference in test-retest reliability was present if conducted in normoxia or hypoxia, two groups of 6 males completed 4 laboratory visits over a 4-week period. One group completed 3 HTPT in normoxia (NORM), the other in moderate hypoxia (HYP; F_IO_2 0.14). These tests would also serve to determine how many familiarization sessions would be required prior to conducting experimental sessions involving the performance test. Participant characteristics are presented in Table 3.11.3. In this instance it was not possible to match pairs based on physiological measurements prior to the testing protocol.

The first session was used to determine lactate threshold, D_{max}, and maximal aerobic capacity as described in section 3.6.1. In this instance an electromagnetically braked cycle ergometer (SRM; scientific model, Julich, Germany) was used for all testing. The SRM scientific model consists of 8 strain gauges, calibrated against unloaded cycling according to the manufacturers guidelines, to ensure an accuracy of ± 0.5%. The standard error of the SRM powermeter slope is 0.01Hz.Nm^{-1}, equating to 1 Watt in 1000 (Wooles et al., 2005). Power output was recorded continuously at a sampling rate of 0.5Hz. Data were recorded onto a Powercontrol IV (SRM; Julich, Germany) and downloaded onto a personal computer and analysed with SRMwin software.

Table 3.11.3. Mean ± SD participant characteristics for normoxia (NORM, *n* = 6) and hypoxia (HYP, *n* = 6). **PPO** = peak power output; **LT1** = Power output at which blood lactate increased 1mmol above the measured resting value; **LT4 =** Power output at which blood lactate reached 4mmol•L^{-1}; Dmax = Power output at which D_{max} was reached.

	Age (yr)	Height (m)	Mass (kg)	$\dot{V}O_2$ peak ($L \cdot min^{-1}$)	PPO (Watts)	LT1 (Watts)	LT4 (Watts)	Dmax (Watts)
NORM	21 ± 2	1.77 ± 0.04	84 ± 17	3.17 ± 0.4	283 ± 23	124 ± 21	181 ± 17	209 ± 35
HYP	27 ± 5	1.76 ± 0.02	72 ± 7.6	3.82 ± 0.6	299 ± 24	155 ± 37	216 ± 32	231 ± 18

Upon arrival to the laboratory participants were seated on the SRM ergometer and fitted with a heart rate monitor (Suunto T6c, Kemple, Finland). A laser Doppler integrating flow probe was attached to the belly of the vastus lateralis muscle on the right leg, and a pneumatic finger cuff applied to the index finger of the right hand (Portapres Model-2, Finapres Medical Systems, Amsterdam). The cuffed finger was positioned level to the aorta via palpation of the third intercostal space, and held in place with an arm sling (Moore & Agur 2007). A height sensor was connected for level correction to calculate blood pressure variations caused by changes in hydrostatic pressure changes above or below the finger cuff. Stroke volume and cardiac output were then derived from the arterial blood pressure waveform. For reproducibility, cuff size and the finger used were the same in each trial to measure arterial waveform. A fingertip capillary blood sample was collected for determination of resting blood lactate, hemoglobin and hematocrit using the methods previously described (Section 3.8). All samples were collected in triplicate, and mean values recorded.

Participants then began a 15-minute resting wash-in period breathing through the gas delivery system. Respiratory gases were collected between minutes 5 and 10, and between 10 and 15 of the wash in period for determination of resting $\dot{V}O_2$, $\dot{V}CO_2$, RER and minute ventilation (BTPS) using standard Douglas bag technique (Section 3.6.1). The tolerance aspect of the test consisted of 40 minutes cycling at 50% of predetermined normoxic $\dot{V}O_{2\ peak}$, determined via the equation derived from the regression line (oxygen consumption plotted against power output). During this period, heart rate, arterial oxygen saturation, respiratory gases and finger-arterial waveform were collected every 5 minutes throughout the test. Measures of blood lactate, hemoglobin, RPE and thermal sensation were collected every 10 minutes, and a final measure of hematocrit was obtained upon completion of the tolerance test. Participants then underwent a 5-minute recovery period. During this time HR, SpO_2, CO, SV, and MAP were

recorded at the end of each minute, and a final Douglas bag and blood lactate sample was collected between minutes 4 and 5. Immediately after minute 5 of recovery the Portapres was removed from the participant to allow free range of movement during the time-trial aspect of the test.

Participants were then instructed to complete a 16.1 km time trial as quickly as possible. The only feedback available to participants was distance completed. All other variables were concealed. The time trial was controlled using the SRMwin 'Open-ended' mode. This mode creates a braking force that has a cubic relationship with speed, mimicking the effect of air resistance on a moving bicycle. During the time trial only measures of HR, power output and cadence were collected. This was to avoid disturbing participant concentration and to avoid providing any external cues that may give participants an indication of time taken. Arterial oxygen concentration was monitored during all hypoxic trials for participant safety. At least 5 days were given as recovery between testing sessions. Time trial reliability was assessed using the coefficient of variation (CV), expressed as the group standard deviation divided by the grouped mean values. This reliability measure allows comparison with other studies utilizing similar performance tests. CV was computed for time to completion, mean power output (MPO) and mean heart rate during the time trial. The typical error of measurement for completion time, MPO, and mean heart rate was also assessed across each time trial (TT1 v TT2, TT1 v TT3, and TT2 v TT3). This was calculated by dividing the standard deviation of the difference score between time trials by $\sqrt{2}$ (Hopkins, 2000).

3.11.2 Physiological responses to the time-trial

Time to complete the 16.1km course was longer in HYP compared to NORM during TT1 (42.28 ± 5.52 Vs 39.37 ± 1.06 minutes), TT2 (41.38 ± 5.47 Vs 39.11 ± 1.52 minutes) and TT3 (41.75 ± 5.33 Vs 38.77 0.61 minutes), although no significant TT x condition effect was observed ($F_{(2,20)}$ 1.36, p = 0.250) The lack of a significant difference between the NORM and HYP groups can likely be explained by the disparity in physiological profiles of the NORM and HYP groups. The HYP group had significantly higher power outputs at LT1 (155 ± 37 Vs 124 ± 20W) LT4 (216 ± 32 Vs 181 ± 17W) and at D_{max} (231 ± 18 Vs 209 ± 35W) compared to the NORM group.

3.11.3 Within subject differences and test – retest reliability

The typical error was calculated between all trials for each group, and both groups combined, for completion time, mean power output, and mean heart rate during the 3 time trials and are presented in Table 3.11.6. The typical error of completion time following one previous experimental visit (trial 1 Vs trial 2) was between 59 -79 seconds and 68 – 92 seconds for the NORM and HYP groups respectively, with a CV of 0.46% and 1.53% (Table 3.11.6). This represents quite a substantial difference between 2 repeated time trials completed in NORM and two repeated in HYP. If two familiarisation visits are used prior to data collection, the typical error or measurement (0.39 mins, NORM and 0.36 mins, HYP) indicate that an observable change in TT time of between 34-46 seconds and 32 – 43 seconds for NORM and HYP respectively. Thus, to establish if a change in time was the result of a genuine physiological change, and not caused by variation and error in the test itself, TT times would need to be different by > 46 seconds. The CV between trial 2 and trials 3 are also similar between NORM (0.62%) and HYP (0.63%). Two familiarization visits are therefore enough to habituate participants to the testing protocols, and to ensure adequate sensitivity of the test

to detect real physiological changes as a result of an intervention. Individual coefficient of variation between trials were also calculated as the mean of two (1 vs 2, 1 vs 3 and 2 vs 3) trials divided by the standard deviation of the two trials being examined. Results are presented in Table 3.11.6. These results indicate that two familiarization sessions are adequate to prepare participants for the experimental HTPT. It is also interesting to note that the CV for completion time between trials was not significantly different whether completed in NORM or HYP. The CV for time to completion, MPO and heart rate during the TT is similar to those reported previously for similar distance time trials (Currell and Jeukendrup, 2008).

Table 3.11.4. Typical error and CV for variables collected during the time trial for N (*n* = 6), HYP (*n* = 6) and combined (*n* = 12)

Variable	Typical error			Coefficient of variation		
	TT1 & TT2	TT1 & TT3	TT2 & TT3	TT1 & TT2	TT1 & TT3	TT2 & TT3
Completion time (Minutes)						
Normoxia	0.66	0.41	0.39	0.46	1.08	0.62
Hypoxia	0.77	0.59	0.36	1.53	0.90	0.63
Combined	0.71	0.48	0.47	1.01	1.12	0.10
Mean power output (Watts)						
Normoxia	5.0	8.8	5.7	0.45	2.56	3.01
Hypoxia	2.3	8.0	6.3	2.30	1.56	0.71
Combined	4.6	8.4	7.7	0.94	2.06	1.12
Mean HR (beats•min^{-1})						
Normoxia	3	4	2	1.56	0.51	1.06
Hypoxia	3	5	3	0.43	0.28	0.71
Combined	3	4	3	0.55	0.09	0.47

3.12. Blood sampling and analysis

3.12.1 Blood Collection

Venous blood samples were drawn by standard venepuncture technique from an antecubital vein, after 10 min in a supine position, into potassium EDTA (mHSP72) vacuette tubes (Vacuette®, Greiner Bio-one, UK). The blood collection timings, i.e. time points, are highlighted in their respective chapters. This consistent timing of blood sample collection was important to account for diurnal and circadian variations in basal *mHSP72* (Chapters 4 and 5), especially as basal values can dictate the magnitude of stressor mediated changes in HSP72, both *in vivo* (Gjovaag and Dahl, 2006) and *in vitro* (Vince et al., 2010).

3.12.2 HSP72 specific blood collection considerations

Fortes and Whitham (2009) demonstrated that repeated venepuncture induced no stress-induced changes in HSP72 in comparison to repeated cannulation blood draws. Therefore, rather than regularly flushing cannulas, which may dilute/alter sample concentration repeated venepuncture blood draws were utilized. EDTA coated tubes were utilized as these have been shown to yield higher HSP72 values than other anti-coagulation tubes, which for low basal HSP72 values is an important factor (Whitham and Fortes, 2006).

3.12.2.1 Intracellular heat stress protein 72

Flow cytometry was used to quantify intracellular HSP72 in peripheral blood mononuclear cells (PBMCs). Whole blood (100µL) from EDTA tubes was transferred into a 2mL red blood cell lysing buffer (Erythrolyse, AbD Serotec, UK) and incubated at room temperature for 10 minutes. The sample was then centrifuged at 500g for 5 minutes and the supernatant discarded. Cells were then washed by adding 2mL of phosphate buffer saline and spun for 5

minutes at 500g. Cells obtained after red cell lysis were fixed for 15 minutes by adding 100 µL of fixative (Reagent A, Leukoperm, AbD Serotec, UK) at room temperature, and after a further wash step, they were permeablised (100µl of Reagent B, Leukoperm, AbD Serotec, UK). A negative control (4µl; FITC, AbD Serotec, UK) or an anti-HSP72 antibody (4µl; SPA-810, Assay Designs, USA) was added to a final concentration of 100ug/ml, to label 1 x 10^6 cells according to the manufacturers instructions, and then incubated for 30 min in the dark. An IgG1 (HSP72) and concentration matched FITC conjugated negative control was used to asses non-specific binding. Samples were then washed with phosphate buffered saline (PBS) before analysis on a BDFACSCalibur (BD Biosciences, UK) by flow cytometry with monocytes gated by forward/side scatter properties and further discriminated by CD14 expression. Mean fluorescence intensity (MFI) was then calculated using CELLQuest software (BD Biosciences, UK) with a total of 15,000 cells counted. Figure 12.1 illustrates a typical forward side scatter showing different cell types, and a frequency histogram for cells stained with a negative and positive HSP72 antibody.

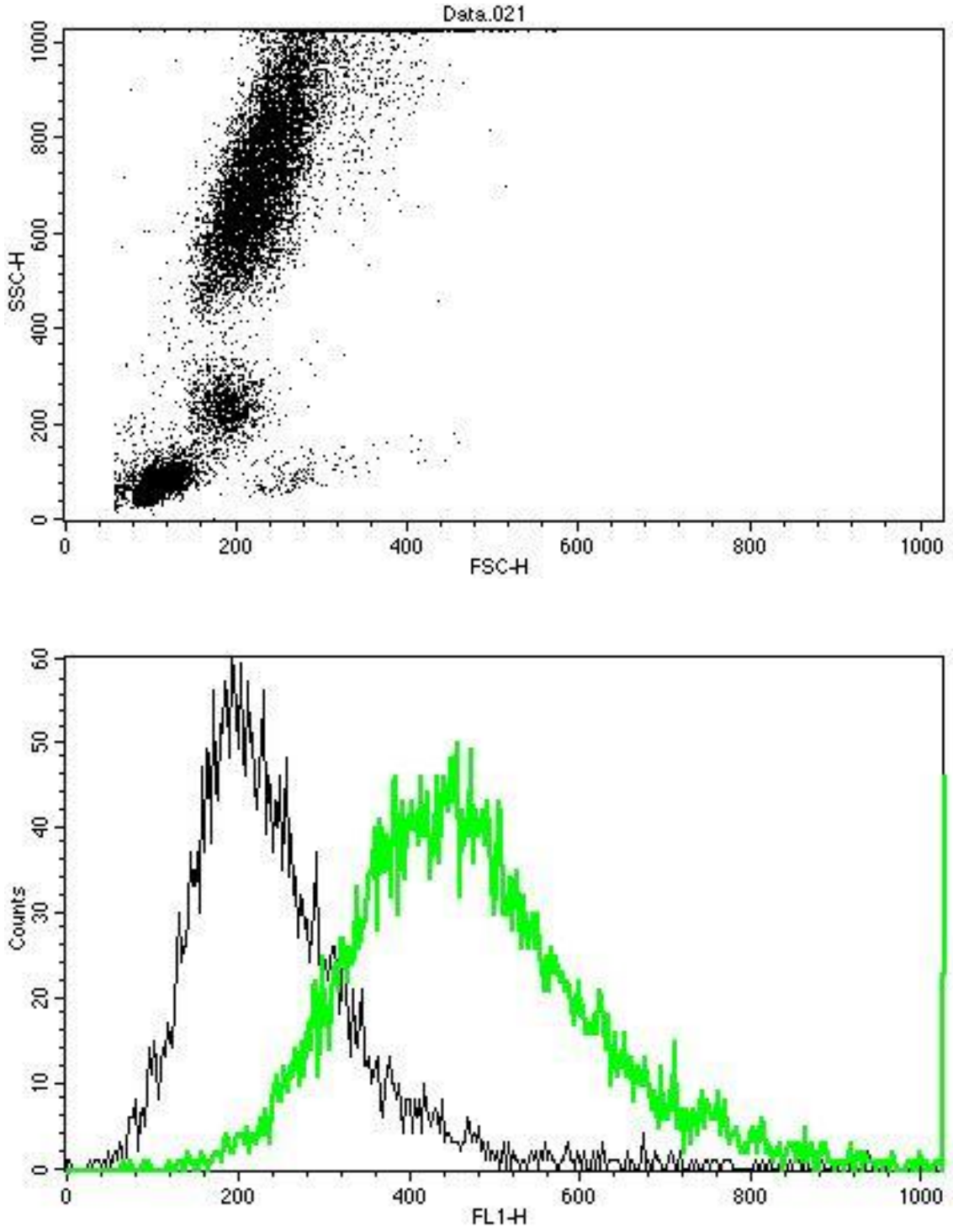

Figure 12.1. Typical flow cytometry profiles, showing **a)** forward scatter (FSC-H)/side scatter (SSC-H) and gated neutrophils, monocytes, and lymphocytes and **b)** fluorescence intensity (FL1-H nm; FITC stained samples on a linear scale) of monocytes incubated with isotype matched negative control (black) and anti-HSP72 (green) antibodies.

It was important to determine the daily variation and reliability of the flow cytometry method of monocyte HSP72 quantification in determining repeated resting values over multiple days without an intervention. Any such variation may be relevant to sporting performance, and in the context of this thesis, the evaluation of efficacy regarding acclimation protocols and preconditioning stressors as mHSP72 was to be used as a marker of cellular tolerance to hypoxic stress and acclimation status.

To determine the reliability and sensitivity of this method 5 males attended the laboratory on 4 occasions, at the same time of day that experimental sessions were scheduled to begin in the experimental chapters (≈10.00am). Samples were collected on a Monday and Tuesday, and then a week later on Monday and Tuesday to simulate the time points to be used in subsequent studies. A one-way repeated measures ANOVA was conducted to determine if there was an observable main effect for time ($F = 0.181$, $P = 0.945$). Table 3.12.1 shows the mean difference between repeated resting measures, 95% confidence intervals, and the coefficient of variation for between each time point. The low CV (< 6%) between resting, untreated samples from participants indicates that this technique is a reliable and sensitive assay for mHSP72 quantification. Studies employing similar protocols have observed an increase in basal mHSP72 in the region of 30% 24 hours after a resting hypoxic exposure (75 minutes at an altitude equivalent to 3000meters above sea level; Taylor et al., 2010), thus the systematic and daily variation in mHSP72 expression would not be expected to confound any true treatment effects in this present series of investigations.

As previous research has reported post exercise increases in intramuscular HSP72 (imHSP72) to persist for 7 days following the exercise bout (Morton et al., 2006), it is feasible that any physical activity 7 days prior to HSP72 sampling may illicit undue effects on basal values and lead to erroneous results. This may also be evident in the blood. However, the

relationship between imHSP72 and PBMC derived HSP72 has not been defined. Thus to determine the reliability and sensitivity of the assay in response to a hypoxic challenge, and to determine whether 7 days between stress exposure is a sufficient washout period, 5 of the participants from Section 3.11 provided blood samples pre and post HST on 3 occasions each separated by at least 7 days.

The coefficient of variation for the resting expression of mHSP72 was similar to that reported in Table 3.12.1 (HST 1 vs HST 2 = 5.59%, HST 1 vs HST 3 = 3.58%, HST 2 vs HST 3 = 5.25%), indicating that a 7 day washout is sufficient between experimental trials to allow for a 'resetting' of the HSP72 response to an exercise challenge. Table 3.12.2 shows the mean difference, 95% confidence interval and coefficient of variation for post exercise mHSP72 and figure 3.12.2 displays the individual resting data for each participant prior to completing each HST.

Table 3.12.1. Paired comparisons at 5 different time points for resting mHSP72 MFI.

Comparison	Mean difference (MFI)	95% Confidence interval (MFI)	CV (%)
Day 1: Day 2	0.03	-0.29, 0.35	4.31
Day 1: Day 3	0.09	-0.39, 0.59	3.58
Day 1: Day 4	0.16	-0.39, 0.78	4.70
Day 2: Day 3	0.06	-0.47, 0.60	5.25
Day 3: Day 4	0.07	-0.72, 0.21	2.53

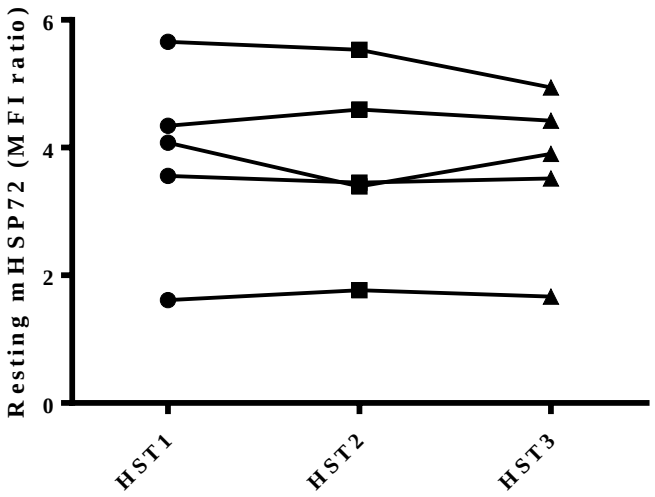

Figure 3.12.2. Resting data for each participant prior to commencing each HST.

The response to an exercising hypoxic challenge was shown to be consistent and repeatable when the pre-experimental conditions, such as time of day of testing, and adequate dietary controls, were tightly controlled. Post exercise mHSP72 values had increased from rest values in all participants, although the magnitude of this response was highly variable. The post HST mHSP72 response was shown to be inversely related to basal mHSP72 in each HST and occurred in a repeatable manner following the 7 day wash-out period (Figure 3.12.3). The inverse relationship between basal HSP72 and its induction post stressor is well reported feature of the HSR (Vince et al., 2010; Taylor et al., 2010, 2011).

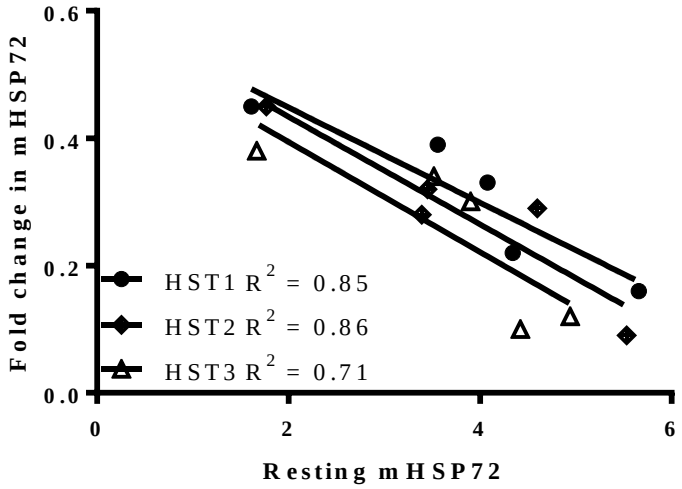

Figure 3.12.3. The mHSP72 response is reliably related to the basal mHSP72 content prior to a HST (n = 5).

Table 3.12.2. Paired comparisons of 3 different time points following three different hypoxic stress tests.

Comparison	Mean difference (MFI)	95% Confidence interval (MFI)	CV (%)
HST 1: HST 2	0.31	-0.81, 1.43	10.3
HST 1: HST 3	0.61	0.19, 1.02	7.97
HST 2: HST 3	0.29	-0.78, 1.38	10.0

The data above have some important implications to consider when designing and implementing the subsequent experimental chapters specific to this thesis. In order to avoid the known influence of basal values on stressor mediated increases in mHSP72, the blood sample collection and scheduling of interventions require careful planning in order to mitigate the known effects of circadian variation on basal values (Taylor et al., 2009, Vince et al., 2010). Thus when attempting to manipulate basal mHSP72 *in vivo* via an environmental

stressor, different interventions must be administered at identical times within the experimental design. This ensures that changes in mHSP72 are due to the intervention and not as a result of circadian variation. Care was therefore taken to schedule all testing sessions at the same time of day for a given participant, and to allow at least 7 days between the end of one experimental trial and the commencement of the next.

3.12.2.2 Circulating heat stress protein 72

Venous blood plasma samples collected during the experiments described in Chapter 5 and Chapter 6 were assayed for eHSP72 using a pre-prepared Enzyme-Linked Immunosorbent Assay (ELISA) kit (StressExpress HSP72 high sensitivity ELISA kit, Stressgen Bioreagents, Victoria, Canada). Plasma was selected over serum because it has been shown to yield higher HSP72 concentrations than serum (Whitham and Fortes, 2006). HSP72 was evaluated via sample absorbance at 450 nm by a microplate reader (ELx800, Bio-Tech Instruments, Inc. Winoski, USA) with software package (KC Junior V1.41.3, Bioteck Instruments, Inc. Winoski, USA). Concentrations of HSP72 were established by plotting recombinant HSP72 standard concentration and absorbance measures on a log log scale to determine a line of best fit. The linear equation generated was then used to gain inducible HSP70 concentration ($ng \cdot mL^{-1}$) from the absorbance of each sample. The sensitivity of the ELISA kit was 0.09 $ng \cdot mL^{-1}$ with both inter and intra-assay coefficient of variation values of less than 10% (StressExpress HSP72 ELISA kit, Stressgen Bioreagents, Victoria, Canada).

3.12.3 Plasma cytokines

Plasma TNF-α, IL-10, and IL-6 was determined independently using enzyme-linked immunosorbant assays (ELISA max, Biolegend, UK) with a sensitivity of 2, 2 and $4pg \cdot ml^{-1}$ respectively. The CV for each assay was determined from analysing duplicate samples (*n* =

24) from each plate and were 6.9%, 5.2% and 8.4% for TNF- α, IL-10 and IL-6 respectively. Data were corrected for any changes in plasma volume.

3.13 Statistical analysis

All data handling was performed in Microsoft Excel 2011. Statistical analysis was performed using SPSS for Mac, version 20 (SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation (SD). The specific statistical procedures employed in each individual experiment are presented in their respective chapter.

3.14 Publications

This chapter has formed the basis of the conference abstracts detailed below:

Lee, B; Emery-Sinclair, E; Mackenzie, RWA; James, RS; Thake, CD (2013). Confirmation of an absolute sub-lactate threshold workload for use in studies combining hypoxia and heat stress. *British Association of Sport and Exercise Scientists Annual conference, Lancaster.*

Lee, B; Owen, R; Emery-Sinclair, E; Mackenzie, RWA; James, RS; Thake, CD (2013). A comparison of physiological responses to prolonged submaximal exercise in individual and combined environmental stressors. *British Association of Sport and Exercise Scientists Annual conference, Lancaster*.

Lee, B; Miller, A; Owen, R; Thake, CD (2013) Comparison of $\dot{V}O_2$ peak between individual and combined environmental stressors. *British Association of Sport and Exercise Scientists Annual conference, Lancaster*.

Chapter 4. The impact of sub-maximal exercise during heat and/or hypoxia on the cardiorespiratory and monocyte HSP72 responses to subsequent (post 24 hr) exercise in hypoxia.

This experimental chapter has formed the basis of the publication detailed below:

B. J. Lee, E. Emery-Sinclair, R. Mackenzie, A. Hussain, L. Taylor, R. S. James & C. D. Thake (2014). The impact of sub-maximal exercise during heat and/or hypoxia on the cardiovascular and monocyte HSP72 responses to subsequent (post 24 hr) exercise in hypoxia. *Extreme Physiology and Medicine, 3*.

4. 1 Introduction

The acute physiological and biochemical responses to the environmental stressors of heat and hypoxia are well characterized when viewed in isolation (Gonzalez-Alonso et al., 2008; Naeije, 2010; Mazzeo, 2008), yet in reality stressors can be and are often experienced in combination. However, few studies have examined the physiological and biochemical effects of such stressors combined (Tipton, 2012).

Acute heat and hypoxic exposures at rest and during exercise produce similar physiological, metabolic and cellular responses (Gonzalez-Alonso et al., 2008; Naeiji, 2010; Horowitz, 2007). For example, heart rate and minute ventilation are elevated in comparison to the same absolute workload under temperate and normoxic conditions. Disturbances to redox balance, seen in response to both heat and hypoxia (Taylor et al, 2010; Taylor et al., 2010) and augmented by exercise, are potent stimuli for increases in heat shock protein concentrations, specifically heat shock protein 72 (HSP72), in humans (Kregel, 2002). This shared and transient response facilitates adaptation to chronic stress (acclimation) and potential cross-tolerance to subsequent diverse stressors (Kuennen et al., 2010; Taylor et al., 2012). In the relative short term, the heat shock response (HSR) can confer tolerance to future exposure to

a stressor(s); this is termed preconditioning (Sharp et al., 2004). Preconditioning documented 1 h after stress insult has been termed "classical preconditioning" and that 1–2 days after stress insult, the "second window of protection" (SWOP) (Taylor et al., 2012). For example, prior exposure to a preconditioning heat stress is known to confer survival to an otherwise lethal heat shock in cell lines (Mizzen and Welch, 1988) and in both tissue-specific and whole-body models in rodents (Landry et al., 1982). In humans, preconditioning may block pro-inflammatory cytokine pathways or alter cellular cytokine tolerance (Amorim and Mosely, 2010). The HSR modulates cytokine signal transduction and gene expression by inhibiting translocation of nuclear factor-kappa B (NF-κB) to the nucleus, thus preventing the activation of the inflammatory cascade and increases in tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (for review, see Amorim and Mosely, 2010). Furthermore, increased expression of heat shock factor 1 (HSF-1) increases the expression of anti-inflammatory interleukin-10 (IL-10; Xiao et al., 2006). Human studies using acute exercise or heat acclimation protocols to increase HSP72 have failed to alter cytokine levels in *ex vivo* heat- or lipopolysaccharide (LPS)-treated cells (Sharp et al., 2004).

Physiological strain drives the adaptive process (Cotter, 2013); thus, determining the magnitude of strain induced by defined levels of heat and hypoxia could potentially inform both training strategies and be used as an adjunct in maintaining and/or aiding the recovery of function from injury. For example, athletes recovering from injury may need to reduce mechanical loading but as a consequence reduce systemic physiological strain limiting the aerobic training stimulus (Linney et al., 2014). The additional imposition of either heat or hypoxia would allow physiological strain to be maintained or increased during rehabilitation/recovery. Furthermore, the characterization of heat and hypoxic responses could also play a role in optimizing the management of movements of individuals or groups

(e.g. military personnel) between different environmental settings. For example, individuals who are physiologically adapted to heat may tolerate moderately hypoxic environments better than non-acclimated individuals (Heled et al., 2012).

To date, no research has compared the physiological, HSP72, and cytokine responses to exercise performed at an absolute work intensity in both heat and hypoxia and the combination thereof. Neither has the impact of this prior exposure on subsequent tolerance to hypoxic exercise been investigated.Therefore, the first aim of this study was to compare the magnitude of physiological and cellular HSP72 and pro/anti-inflammatory cytokine responses to individual and combined exposures to heat and hypoxia during prolonged moderate intensity exercise in young, moderately fit, non-cycle-trained adult males. It was hypothesized that the combination of heat and hypoxia would increase physiological and cellular strain when compared to the individual stressors alone and that greater physiological strain would produce an enhanced heat shock response. The second aim was to determine how the prior exposure to heat and hypoxia alone or in combination would impact upon the physiological and cellular responses to a subsequent hypoxic exposure, 24 h after this initial exercise bout. It was hypothesized that inducing the greatest levels of physiological strain and heat shock response after the initial exposure would enhance physiological and cellular tolerance to hypoxia 24 h later in the participant population studied.

4. 2 Methods

Twelve healthy male participants (mean ± standard deviation: age 22 ± 4yr, height 1.77 ± 0.05m, mass 79.0± 12.9kg, estimated body fat 13.7 ± 4.3%, normoxic $\dot{V}O_{2\ peak}$ 3.57 ± 0.70 $L \cdot min^{-1}$) volunteered to take part in this study, which was given ethical approval by Coventry University Ethics Committee. Participants attended the laboratory on 9 separate occasions.

The initial visit involved preliminary tests for resting hemoglobin concentration and anthropometry to estimate body fat (Durnin and Womersley 1974) followed by the measurement of lactate threshold and peak oxygen uptake ($\dot{V}O_2$ peak) as described in Section 3.6.1.

4. 2. 1 Experimental protocol

Participants were exposed to four experimental trials; – normothermic normoxia (NORM; 20°C, 40% relative humidity RH); heat (HEAT; 40°C, 20% RH), hypoxia; (HYP; F_IO_2 0.14%, equivalent to 3095m, 20°C, 40% RH) and heat and hypoxia combined (COM; F_IO_2 = 0.14, 40°C, 20% RH) using a randomized block design. Participants sat within the defined environment for 30 minutes followed by 90 minutes of submaximal cycling exercise at 50% normoxic $\dot{V}O_2$ peak. Pilot work demonstrated that this absolute workload remained below lactate threshold in normoxia and hypoxia for the 90-minute duration, and below the lactate threshold during the combined trial (Section 3.10; Lee et al. 2013). 24 hours post-trial participants undertook a standardized hypoxic stress test (HST) at a simulated altitude of 3095m above sea level (HST trials 24 hours after NORM, HEAT, HYP and COM are referred to as HST_{NORM}, HST_{HEAT}, HST_{HYP} and HST_{COM} respectively). Details of the experimental method and timings of measurements throughout this investigation can be seen in Figure 4.1.

On each occasion participants provided a urine sample for the assessment of urine specific gravity (USG; Visual refractometer, Index Instruments, Cambridge, Cambridgeshire, UK) and urine osmolality (Osmocheck, Vitech Scientific, Partridge Green, West Sussex, UK), weighed themselves nude to ± 0.1 kg, and inserted a rectal thermometer (Grant Instruments, Cambridge, UK) 10cm past the anal sphincter. A heart rate monitor (Suunto T6c, Suunto,

Finland) was fitted around the chest. Arterial hemoglobin (Hb) saturation (S_PO_2) was monitored throughout and recorded during respiratory gas collections using a finger-clip pulse oximeter sensor fitted around the wrist (3100 WristOx, Nonin Medical Inc., Plymouth, USA). The wrist sensor has a reported accuracy of ± 2 digits (manufacturers guide). Whilst seated, skin thermistors (Grant Instruments) were micropore taped to the upper arm, upper thigh, chest and calf to allow continuous monitoring of mean skin temperature (Ramanathan 1964). During all trials subjects breathed through a mouthpiece and 30 mm diameter connector (Harvard Ltd, Eldenbridge, Kent, UK) attached to a two-way non-rebreathable valve (Harvard Ltd, Eldenbridge, Kent, UK). Ethylene clear vinyl tubing was used to connect the inspiratory side of the valve to a series of 1000L Douglas bags. During normoxic trials the valve was left open to the ambient air. During all hypoxic trials the 1000L Douglas bags were filled with hypoxic gas generated by an oxygen filtration device (Hypoxico HYP123 hypoxicator, New York, USA) prior to the start of all testing.

Expired gas was collected into Douglas bags for 60 seconds every 10 minutes. Cardiac output ($\dot{Q}$), stroke volume (S.V) and a - $\bar{v}O_2$ difference were estimated according to the equation of Stringer et al., (1997). After each expired gas collection participants reported overall perceived exertion and thermal sensation. The physiological strain index (PSI) was calculated as described in Section 3.6.1.

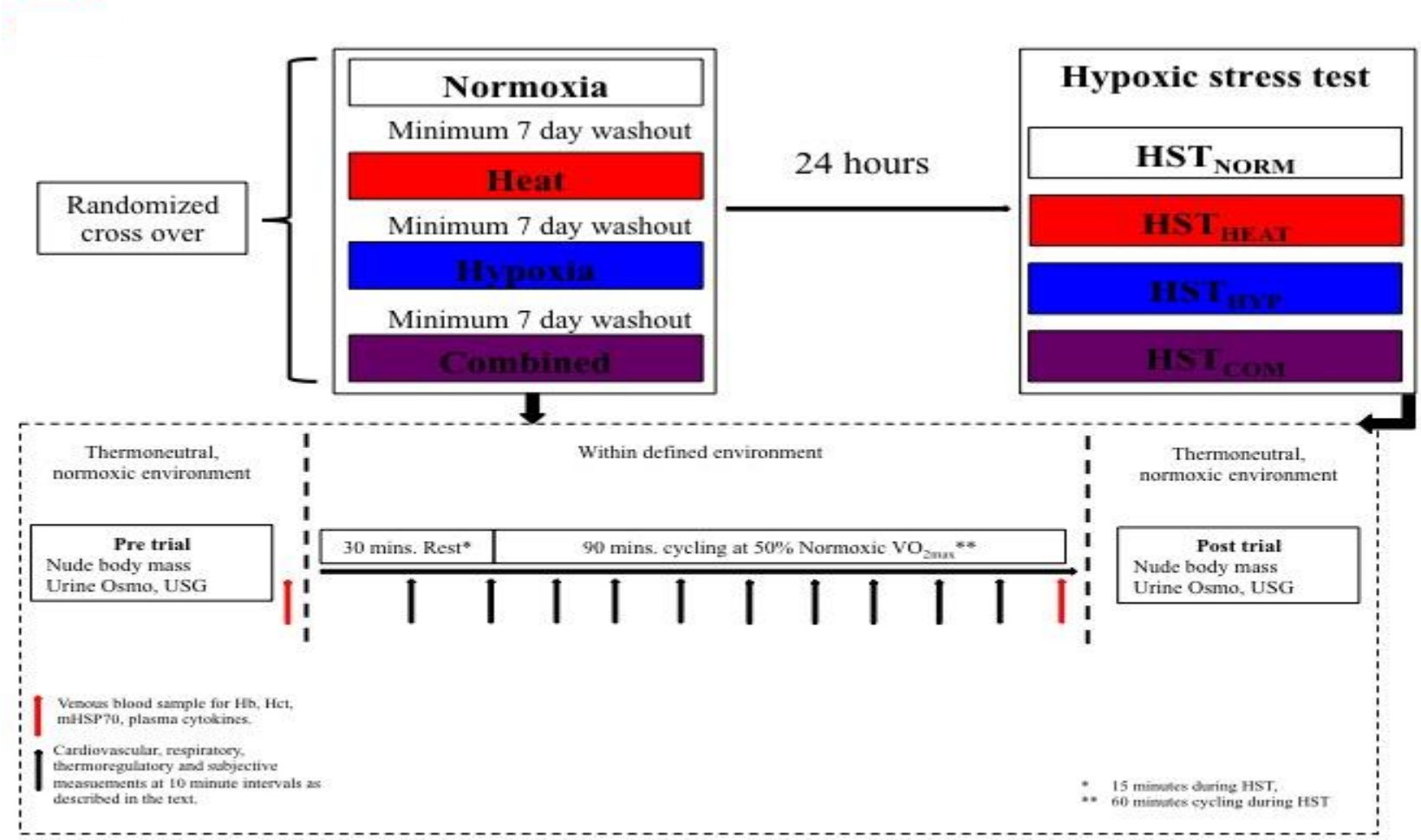

Figure 4. 1. Experimental design and data collection timings during the initial environmental exposure and the subsequent hypoxic stress test 24 hours later.

Venous blood was collected from an antecubital vein into potassium EDTA vacutainers (Vacuette®, Greiner BIO-one, UK) according to the criteria described in Section 3.12.1 immediately prior to entering the defined environment, and immediately upon cessation of exercise in each experimental session. Venous blood samples were subsequently used for the assessment of monocyte heat shock protein 72 (mHSP72, section 3.12.1), TNF-alpha, IL-10 and IL-6 (3.12.3) Measurements of hematocrit and hemoglobin were made to determine plasma volume according to the methods of Dill and Costil (1974).

4. 2. 2 Hypoxic stress test

Participants returned to the laboratory 24 hours after cessation of each experimental trial to undergo a normothermic, hypoxic stress test in order to determine whether the prior acute exposure to NORM, HEAT, HYP or COM conferred any detectable preconditioning effect. Details of this test are in Section 3.11.

4. 2. 3 Statistical analysis

Data were checked for normal distribution prior to analysis. Sphericity was checked with Mauchly's sphericity test, and when necessary the Huynt-Feltd method was applied to the F-ratio to correct for sphericity violations. All data are presented as mean ± SD for $n = 12$, with statistical significance set at $P < 0.05$.

4. 2. 3. 1 Initial environmental exposure and hypoxic stress test

Two-way repeated measures ANOVAs (condition by time) were performed to determine differences between environmental conditions both throughout rest and during exercise. Resting data were analyzed separately from exercise data. Exercise data were comprised of measurements made at 10, 20, 30, and 40 minutes, with the final value recorded upon

cessation of exercise (5 time points) for both the initial environmental exposure and the post 24 hour HST. Data were further explored by comparing the percentage change in physiological data collected during HST_{NORM} with all other experimental HSTs via two-way repeated measures ANOVAs (condition by time) and main effects further explored using Tukey's HSD test. Interaction effects are reported when observed. Significant differences between data points were identified using both orthogonal contrasts (data reported *one–tailed, F ratio, P value*), with data obtained during the NORM trial used as reference values, and Tukey's Honestly Significant Difference *post hoc* test (*two-tailed*, reported as $P < 0.05$, < 0.01). Alterations in mHSP72, plasma TNF-alpha, plasma IL-6, and plasma IL-10 were analyzed via a two way repeated measures ANOVA, with main effects ident using Tukey's HSD test. mHSP72 was analyzed as a percentage change from each trial's initial baseline value obtained on day 1 (Taylor et al. 2012).

4. 3 Results

4. 3. 1 Hydration state

All participants were euhydrated prior to the start of each experimental trial, with USG < 1.020 and $U_{osmo} < 300$ mOsmol/kg. Nude body mass did not vary prior to any experimental condition on day 1 (NORM 79.2 ± 13.8 kg, HEAT 79.2 ± 12.8 kg, HYP 79.3 ± 13.8 kg, COM 79.3 ± 13.1 kg).

4. 3. 2 Cardiorespiratory responses at rest

The only physiological variables altered by the resting environmental exposures were heart rate, SpO_2, respiratory exchange ratio (RER), T_{skin} and T_{body}. All resting cardiovascular, respiratory and thermoregulatory data is presented in Tables 4.1 and 4.2.

Table 4.1. Mean ± SD resting cardiovascular and metabolic responses to acute physiological stressors. a = P < 0.05 compared to the corresponding time point in the normoxic condition (*n* = 12).

	BASELINE	15 MIN	30 MIN		15 MIN	30 MIN
Heart rate (beats•min^{-1})				$\dot{V}CO_2$ (L•min^{-1} STPD)		
Normoxia	69 ± 10	65 ± 10	64 ± 10	Normoxia	0.32 ± 0.08	0.30 ± 0.07
Heat	71 ± 14	73 ± 12[a]	75 ± 13[a]	Heat	0.31 ± 0.07	0.32 ± 0.10
Hypoxia	63 ± 9	69 ± 7	74 ± 8	Hypoxia	0.33 ± 0.06	0.35 ± 0.07
Combined	68 ± 9	80 ± 12[a]	82 ± 10[a]	Combined	0.34 ± 0.07	0.33 ± 0.08
S_PO_2 (%)				$\dot{Q}$ (L•min^{-1} STPD)		
Normoxia	98 ± 1	97 ± 1	97 ± 2	Normoxia	5.6 ± 1.3	5.4 ± 0.8
Heat	98 ± 1	97 ± 1	97 ± 1	Heat	5.6 ± 1.0	5.9 ± 1.6
Hypoxia	98 ± 1	90 ± 2[a]	89 ± 3[a]	Hypoxia	5.4 ± 1.1	5.7 ± 1.0
Combined	97 ± 1	91 ± 2[a]	89 ± 3[a]	Combined	5.5 ± 1.3	5.7 ± 1.6
$\dot{V}_E$ (L•min^{-1} BTPS)				Stroke volume(mL•beat^{-1})		
Normoxia	-	13.8 ± 4.4	13.3 ± 2.8	Normoxia	87 ± 18	86 ± 13
Heat	-	13.8 ± 3.7	14.4 ± 4.7	Heat	78 ± 12	79 ± 24
Hypoxia	-	15.0 ± 4.3[a]	14.5 ± 3.6	Hypoxia	80 ± 18	78 ± 19
Combined	-	13.7 ± 2.9	13.1 ± 2.8	Combined	70 ± 15	68 ± 16
$\dot{V}_E$ (L•min^{-1} STPD)				$a - \bar{v}O_2$ difference (mL•L^{-1})		
Normoxia	-	11.3 ± 3.6	10.8 ± 2.2	Normoxia	6.8 ± 0.5	6.8 ± 0.2
Heat	-	11.3 ± 3.0	11.7 ± 3.8	Heat	6.9 ± 0.2	6.9 ± 0.3
Hypoxia	-	12.7 ± 4.8	12.7 ± 3.9	Hypoxia	6.8 ± 0.3	6.9 ± 0.4
Combined	-	11.5 ± 2.7	11.0 ± 2.7	Combined	6.8 ± 0.2	6.9 ± 0.3
$\dot{V}O_2$ (L•min^{-1} STPD)				RER		
Normoxia	-	0.39 ± 0.11	0.37 ± 0.06	Normoxia	0.83 ± 0.07	0.82 ± 0.15
Heat	-	0.39 ± 0.07	0.41 ± 0.12	Heat	0.79 ± 0.10	0.79 ± 0.10
Hypoxia	-	0.37 ± 0.08	0.39 ± 0.08	Hypoxia	0.90 ± 0.09[a]	0.88 ± 0.07[a]
Combined	-	0.38 ± 0.10	0.39 ± 0.13	Combined	0.89 ± 0.08	0.85 ± 0.09

Table 4.2. Mean ± SD resting thermoregulatory measurements. a = P < 0.05 compared to NORM and HYP at the corresponding time point (*n* = 12).

	BASELINE	15 MIN	30 MIN
Core temp (°C)			
Normoxia	37.2 ± 0.3	37.2 ± 0.2	37.2 ± 0.2
Heat	37.2 ± 0.3	37.2 ± 0.4	37.3 ± 0.3
Hypoxia	37.3 ± 0.3	37.2 ± 0.2	37.1 ± 0.2
Combined	37.3 ± 0.3	37.3 ± 0.3	37.3 ± 0.3
Mean skin temp (°C)			
Normoxia	31.1 ± 0.6	31.2 ± 0.9	31.2 ± 0.8
Heat	32.2 ± 0.7	34.9 ± 0.5 [a]	34.9 ± 0.5 [a]
Hypoxia	31.0 ± 1.0	31.2 ± 0.9	31.1 ± 1.0
Combined	31.6 ± 0.7	34.3 ± 1.1 [a]	34.5 ± 1.1 [a]
Mean body temp (°C)			
Normoxia	35.9 ± 0.2	35.9 ± 0.2	35.9 ± 0.2
Heat	36.1 ± 0.3	36.7 ± 0.3 [a]	36.8 ± 0.2 [a]
Hypoxia	36.1 ± 0.2	36.1 ± 0.2	35.9 ± 0.3
Combined	36.1 ± 0.2	36.7 ± 0.3 [a]	36.7 ± 0.3 [a]

When participants were separated into trained (> 50ml•kg^{-1}•min^{-1}, 55.8 ± 5.5ml•kg^{-1}•min^{-1}, *n* = 6) and untrained (< 40ml•kg^{-1}•min^{-1}, 38 ± 2.4ml•kg^{-1}•min^{-1}, *n* = 6) the effects of aerobic fitness became more apparent. The trained group completed 90 ± 0, 80 ± 12, 90 ± 0 and 87 ± 8 minutes of exercise in NORM, HEAT, HYP and COM, whereas the untrained group completed 88 ± 4, 77 ± 12, 72 ± 13 and 60 ± 16 minutes of exercise in NORM, HEAT, HYP and COM. Participant drop out times are shown in Figure 4. 3. Pearson correlations, adjusted for multiple comparisons, revealed that maximal aerobic capacity was positively related to performance time in the HYP (r = 0.699, P = 0.01) and COM (0.598, P = 0.04) conditions, but no such relationship existed for HEAT (r = -0.027. P = 0.933). Table 4.3 shows all physiological values upon termination of exercise in each condition. At the end of exercise

the percentage of normoxic $\dot{V}O_2$ peak was 57 ± 14, 60 ± 9, 59 ± 15 and 57 ± 11% in NORM, HEAT, HYP, and COM respectively.

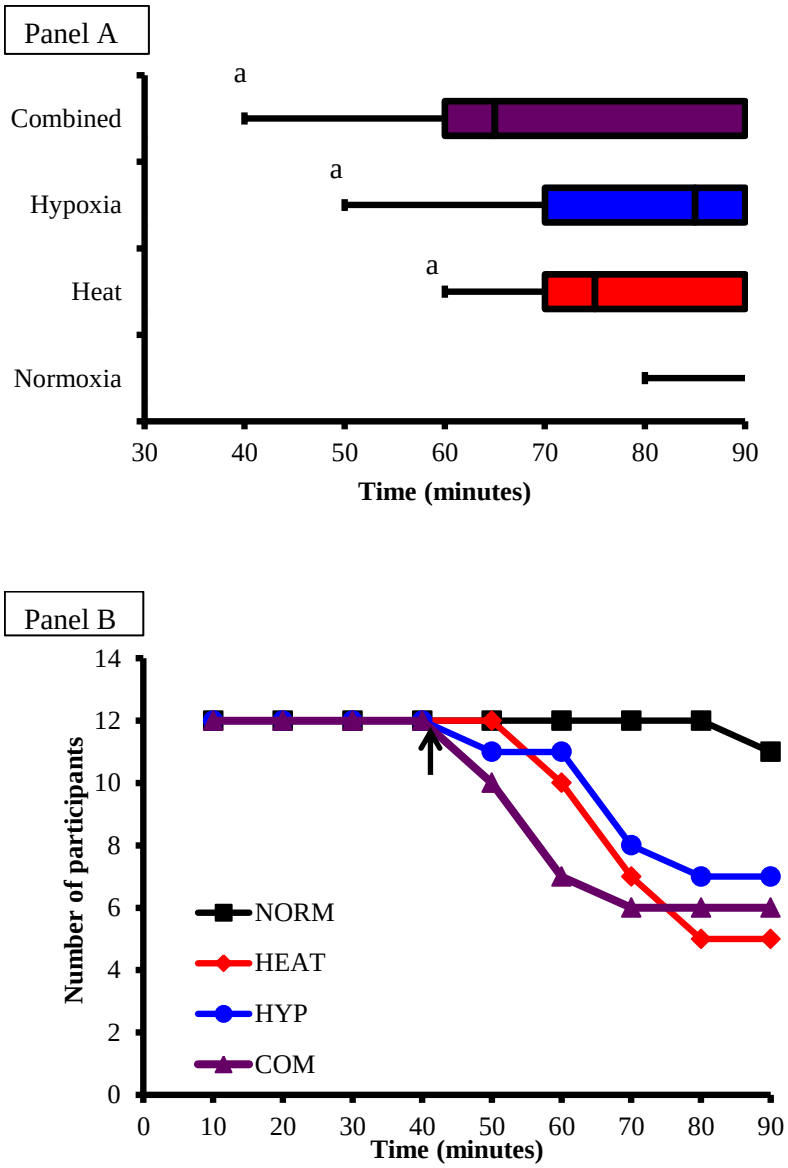

Figure 4.2. Panel A displays exercise times until exhaustion during the 4 experimental trials. Data show the 1st, 2nd (median) and 3rd interquartile range (colored boxes) and shortest achieved time in each condition. *a* = significantly different from NORM ($P < 0.01$). Panel B displays the participant drop-out during each condition. The arrow denotes the point at which pairwise comparisons were made for statistical analysis between the 12 participants.

4. 3. 4. 1 Cardiorespiratory responses to exercise

Heart rate varied between conditions throughout exercise ($F = 2.452$, $P = 0.006$, condition x time interaction) and was lowest in NORM ($P < 0.01$ vs. HEAT, HYP and COM) and tended to be greatest in COM ($P < 0.05$ vs. HYP). HR did not vary between HEAT and HYP until termination of exercise, where HR was higher in the HEAT ($P < 0.05$) and COM ($P < 0.01$) compared with HYP (Figure 4. 3). During exercise SpO_2 was significantly lower at each time point in HYP and COM compared to NORM and HEAT ($P < 0.01$). Upon termination of exercise SpO_2 was significantly lower in HYP and COM compared to NORM and HEAT ($P < 0.01$; Table 4.3). No difference between HYP and COM was found at any time point (Figure 4. 3).

No significant main effect for condition was found for $\dot{V}O_2$ ($F = 0.244$, $P = 0.879$) or $\dot{V}CO_2$ ($F = 1.607$, $P = 0.207$). RER was higher in HYP compared to NORM ($P < 0.01$) and HEAT ($P < 0.01$). RER was higher at the end of exercise in HYP and COM compared to NORM and HEAT ($P < 0.05$). A main effect for condition was found for $\dot{V}_E$ BTPS ($F = 10.685$, $P < 0.001$). $\dot{V}_E$ BTPS was higher in HYP and COM compared to NORM. There was a trend for $\dot{V}_E$ BTPS to be higher in COM compared to HEAT ($P = 0.064$). $\dot{V}_E$ BTPS was higher at the end of exercise in HYP and COM compared to NORM though this did not reach statistical significance ($P = 0.10$).

Table 4.3. Mean ± SD cardiovascular, metabolic, thermoregulatory and subjective data upon termination of exercise across experimental conditions (n = 12). a = significantly different from NORM; b = significantly different from HYP; c = significantly different from HEAT, ($P < 0.05$).

	NORMOXIA	HEAT	HYPOXIA	COMBINED
Cardiovascular				
HR (beats•min^{-1})	138 ± 17	168 ± 15$^{a, b}$	156 ± 13^{a}	174 ± 5$^{a, b}$
SpO_2(%)	96 ± 2	95 ± 1	83 ± 1^{a,c}	83 ± 1^{a,c}
$\dot{Q}$ (L•min^{-1})	16.9 ± 2.5	17.6 ± 2.8	17.3 ± 2.5	17.4 ± 3.2
Stroke volume (ml•bt^{-1})	126 ± 33	106 ± 18	112 ± 23	101 ± 16
a − $\bar{v}O_2$ difference	10.95 ± 2	11.96 ± 1	11.97 ± 1.5	11.7 ± 1.2
Plasma volume change (%)	-2.0 ± 5.8	-3.2 ± 10	-1.7 ± 7.3	-2.4 ± 5.4
Metabolic				
$\dot{V}O_2$ (L•min^{-1})	1.98 ± 0.37	2.09 ± 0.33	2.06 ± 0.42	2.02 ± 0.49
$\dot{V}CO_2$ (L•min^{-1})	1.71 ± 0.34	1.80 ± 0.29	1.91 ± 0.36	1.83 ± 0.35
RER	0.87 ±0.09	0.86 ± 0.06	0.93 ± 0.07^{a,c}	0.92 ± 0.09^{a,c}
$\dot{V}_E$ STPD (L•min^{-1})	40.5 ± 5.90	46.4 ± 9.30^{a}	53.0 ± 12.2^{a}	52.2 ±10.70^{a}
$\dot{V}_E$ BTPS (L•min^{-1})	49.6 ± 7.30	56.9 ± 11.8^{a}	63.3 ± 14.1^{a}	62.1 ± 9.90^{a}
Thermoregulatory				
T_{core} (°C)	37.8 ± 0.3	38.7 ± 0.5^{a,b}	38.0 ± 0.3	38.6 ± 0.4^{a,b}
T_{skin} (°C)	31.4 ± 2.0	36.2 ± 0.9^{a,b}	32.1 ± 1.3	35.8 ± 1.0^{a,b}
T_{body}(°C)	36.5 ± 0.6	38.1 ± 0.4^{a,b}	36.9 ± 0.3	38.1 ± 0.4^{a,b}
Sweat rate (L•min^{-1})	0.48 ± 0.2	1.05 ± 0.2^{a,b}	0.46 ± 0.2	0.91 ± 0.2^{a,b}
PSI (A.U)	4.5 ± 0.9	7.6 ± 1.5^{a,b}	5.2 ± 0.7	7.6 ± 1.1^{a,b}
Perceptual				
RPE (A.U)	14 ± 2	17 ± 2^{a}	17 ± 2^{a}	17 ± 2^{a}
TS (A.U)	5 ± 1	7 ± 1	6 ± 1	7 ± 1

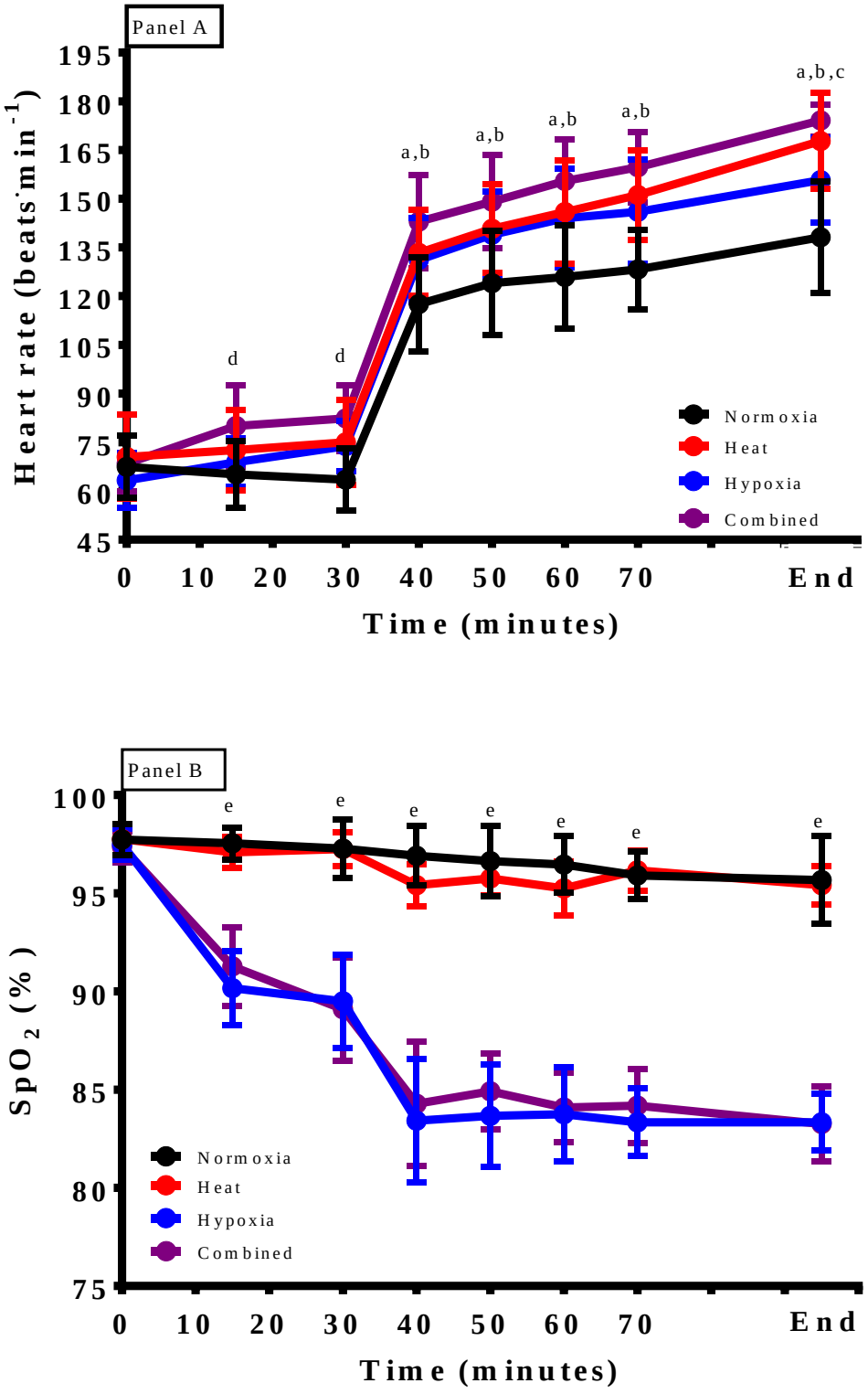

Figure 4. 3. Panel A shows the heart rate response during each trial; Panel B shows the S_PO_2 response during each trial. Data are mean ± SD for *n* = 12. *a* = HEAT, HYP and COM

different compared to NORM ($P < 0.05$); *b* = COM different to HYP ($P < 0.05$); *c* = HEAT different to HYP ($P < 0.05$), *d* = COM different to NORM ($P < 0.05$), *e* = HYP and COM different to NORM and HEAT ($P < 0.01$).

4. 3. 1. 2. Thermoregulatory responses and physiological strain index

T_{core} increased throughout exercise, and was significantly elevated upon exercise termination in all trials (main effect for time; $F = 59.1$, $P < 0.001$). T_{core} was greater at each time point in HEAT and COM compared to NORM and HYP (condition x time interaction; $F = 6.99$, $P < 0.001$; Figure 4.4). During NORM and HYP, T_{core} rose during the initial 20 minutes of exercise before reaching a plateau at 30 minutes. Greater differences between conditions were observed for mean skin temperature, which was higher at each time point, and upon exercise termination in HEAT and COM compared to NORM and HYP (condition x time interaction; $F = 2.478$, $P = 0.006$; Figure 4. 4). Mean body temperature was higher at each time point throughout exercise and upon the cessation of exercise in HEAT and COM compared to NORM and HYP (condition x time interaction; $F = 3.515$, $P < 0.001$). Physiological strain was higher throughout exercise in HEAT, HYP and COM compared to NORM ($P < 0.05$) and all higher than NORM upon exercise termination ($P < 0.01$). Compared to HYP, PSI was higher throughout exercise in the COM trial ($P < 0.01$) and higher during the HEAT trial from 30 minutes through to exercise termination ($P < 0.01$) (Figure 4. 4). Sweat rates and percent change in body mass were higher after exercise in both hyperthermic trials compared to NORM or HYP ($P < 0.001$). Plasma volume did not vary at rest ($F = 1.784$, $P = 0.169$) or post exercise ($F = 1.913$, $P = 0.147$) between trials (Table 4.3).

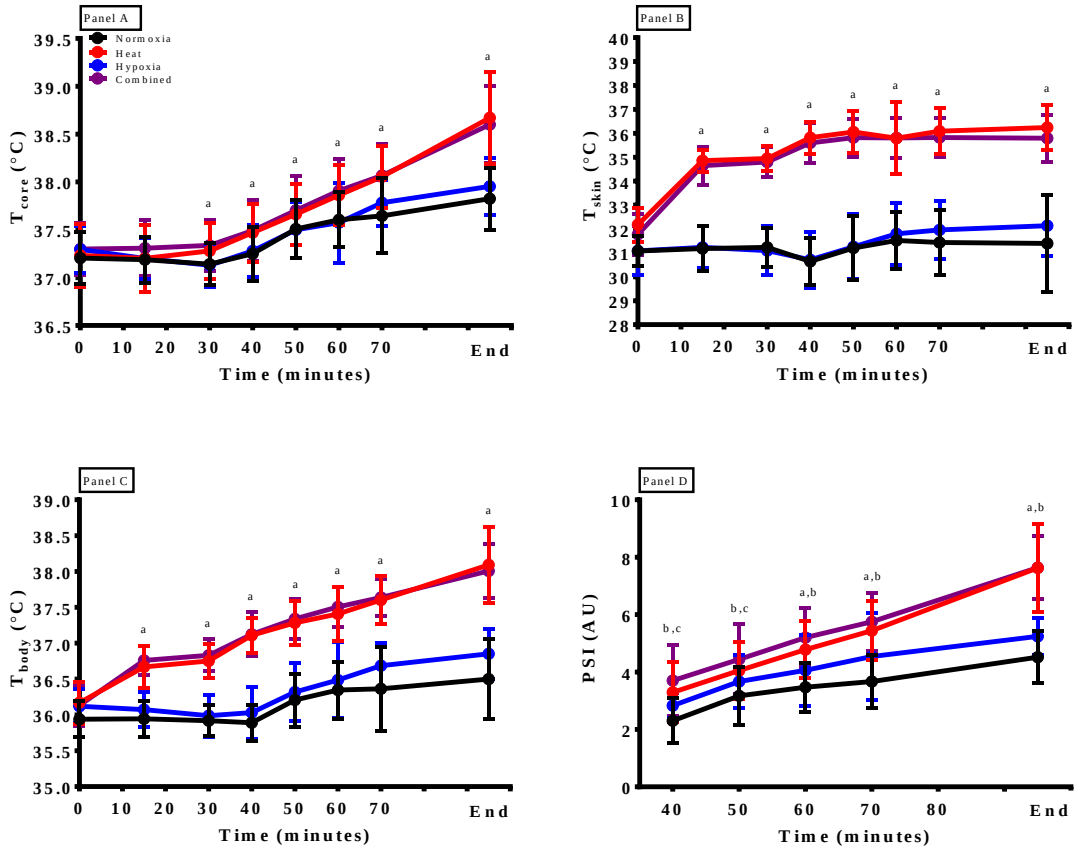

Figure 4. 4. Core temperature (panel A), mean skin temperature (panel B), mean body temperature (panel C) and physiological strain (panel D) increased with time during all trials. Data are mean ± SD for n = 12 The magnitude of increase was greater in the HEAT and COM trials. *a* = HEAT and COM different to NORM and HYP ($P < 0.01$); *b* = HEAT, HYP and COM different to NORM ($P < 0.05$); *c* = COM different to HYP ($P < 0.05$).

4. 3. 1. 3 Ratings of perceived exertion and thermal comfort

RPE increased in a linear fashion throughout all of the trials and was higher throughout exercise in COM compared to NORM and HYP at 10 and 20 min ($P < 0.05$). RPE was significantly higher at the end of exercise in all experimental conditions compared to NORM ($P < 0.01$, Table 4.3), however no difference was found between the other environmental stressors upon exercise termination ($P > 0.05$). Thermal sensation was higher at all time points in HEAT, HYP and COM compared to NORM ($P < 0.01$; Table 4.3). Upon exercise termination thermal sensation was significantly higher in HEAT ($P < 0.01$), HYP ($P < 0.05$), and COM ($P < 0.01$) compared to NORM, and higher in HEAT and COM compared to HYP ($P < 0.01$).

4. 3. 1. 4. Monocyte HSP72 responses to acute environmental exposure

There was no difference between conditions in resting mHSP72 prior to exposure to any experimental conditions. mHSP72 was significantly elevated from rest following exposure to each environmental stressor (Figure 4.5; $P < 0.01$), but was not found to have significantly increased from resting values after NORM (condition x time interaction; $F = 15$, $P = < 0.001$). Post exercise values were higher following HEAT and COM compared to HYP ($P < 0.01$). Post exercise mHSP72 did not vary between HEAT and COM. Post exercise mHSP72 was not related to final core temperature in NORM ($r = -0.214$ $P = 0.505$) and HEAT ($r = 0.199$, $P = 0.536$), whereas a relationship between final core temperature and mHSP72 was observed in HYP ($r = 0.562$, $P = 0.057$) and COM ($r = 0.539$, $P = 0.071$).

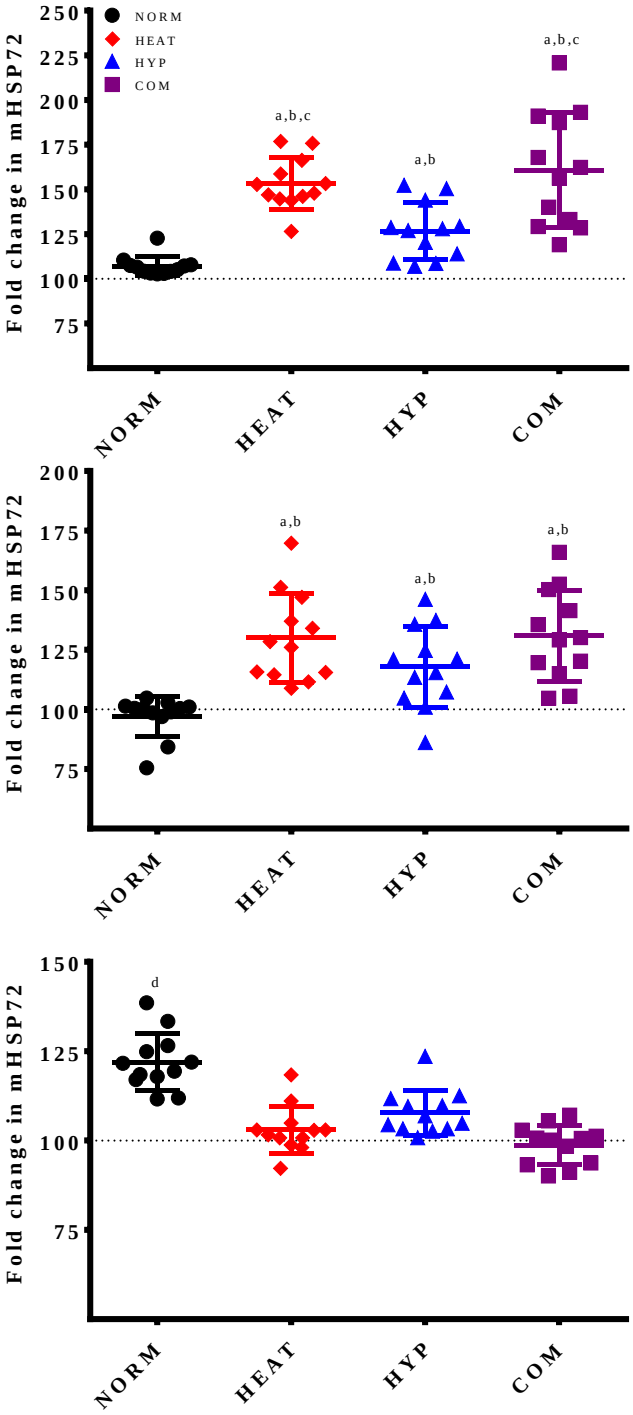

Figure 4.5. Monocyte HSP72 responses after each experimental condition on day 1 (Panel A), pre HST mHSP72 in relation to baseline (Panel B), and fold change in mHSP72 post HST relative to pre HST values. mHSP72 was increased to the greatest magnitude post HEAT and COM exercise and remained elevated 24hrs later. a = difference from baseline ($P < 0.05$), b = difference from NORM ($P < 0.05$), c = difference from HYP ($P < 0.05$), d = difference from pre HST to post HST. Data show each individual participant ($n = 12$). Basal mHSP72 is indicated by the dotted line in panel A and B. In panel C the dotted line illustrates pre HST mHSP72.

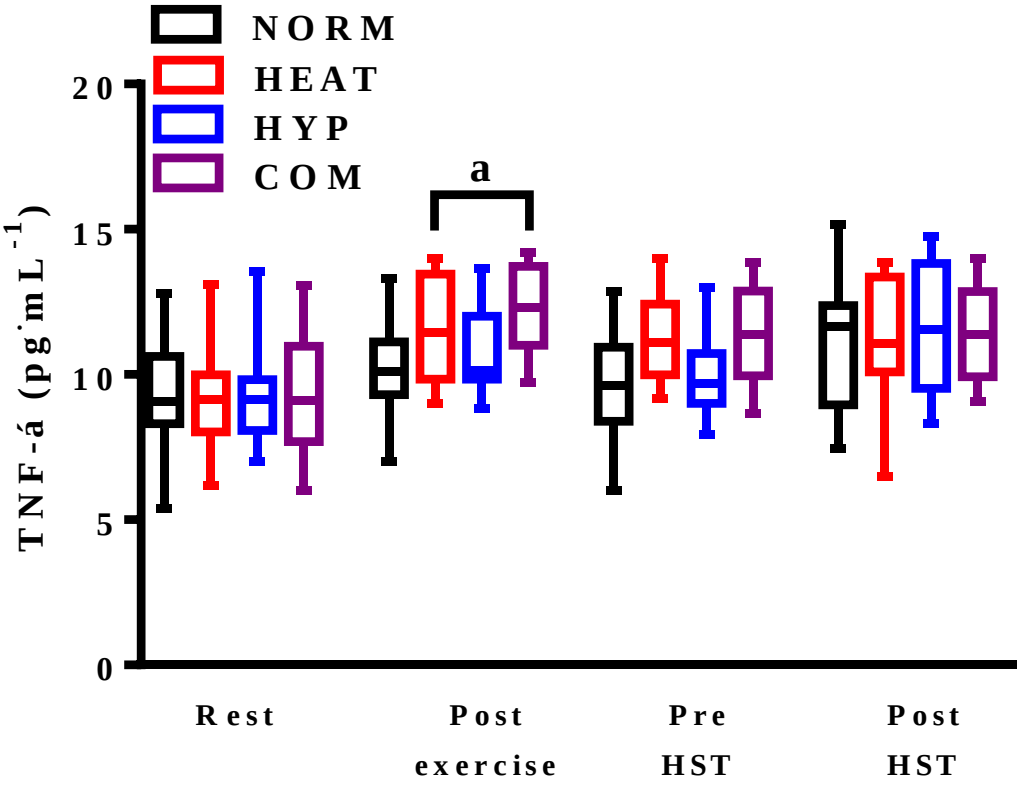

Figure 4.6. Plasma TNF-α at rest, after exercise in each condition, 24 hours after exercise and following the HST (*n* = 12). TNF-α was elevated after exercise in each environmental stressor (P < 0.5). Data show the 1st, 2nd (median) and 3rd interquartile range (coloured boxes) and error bars represent the range from highest to lowest data point achieved time in each condition.

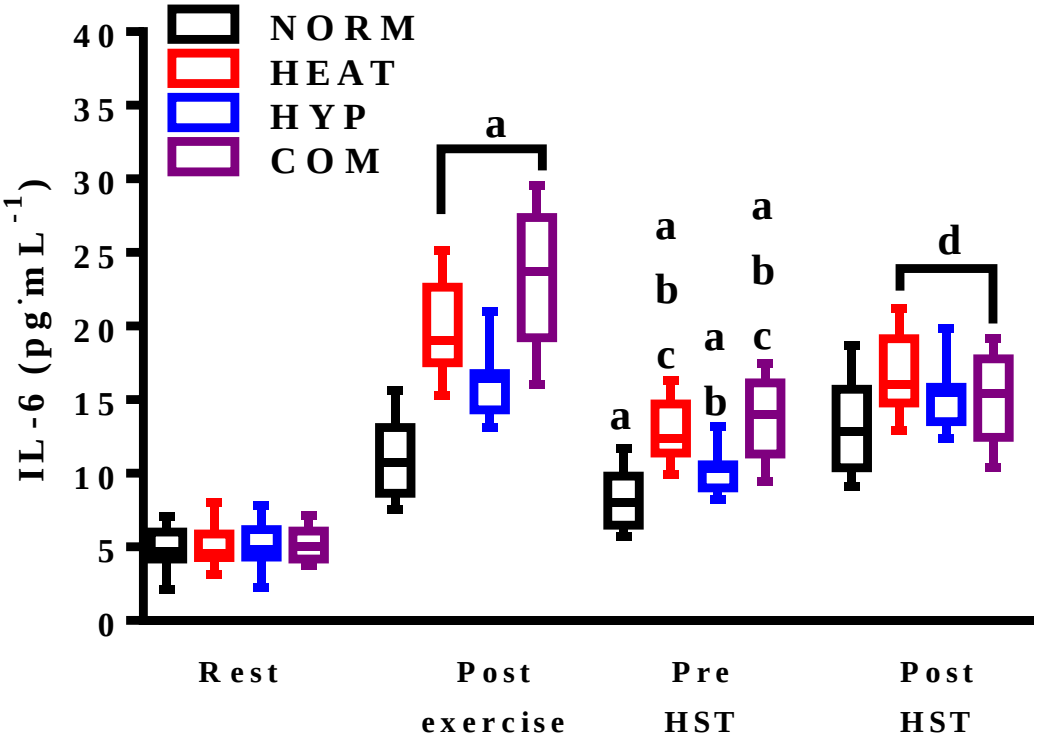

Figure 4.7. Plasma IL-6-α at rest, after exercise in each condition, 24 hours after exercise and following the HST IL-6 increased post exercise in all trials and to the greatest magnitude in HEAT and COM (*n* = 12). a = difference from baseline (P < 0.05), b = difference from NORM (P < 0.05), c = difference from HYP (P < 0.05), d = difference from pre HST to post HST. Data show the 1st, 2nd (median) and 3rd interquartile range (coloured boxes) and error bars represent the range from highest to lowest data point achieved time in each condition.

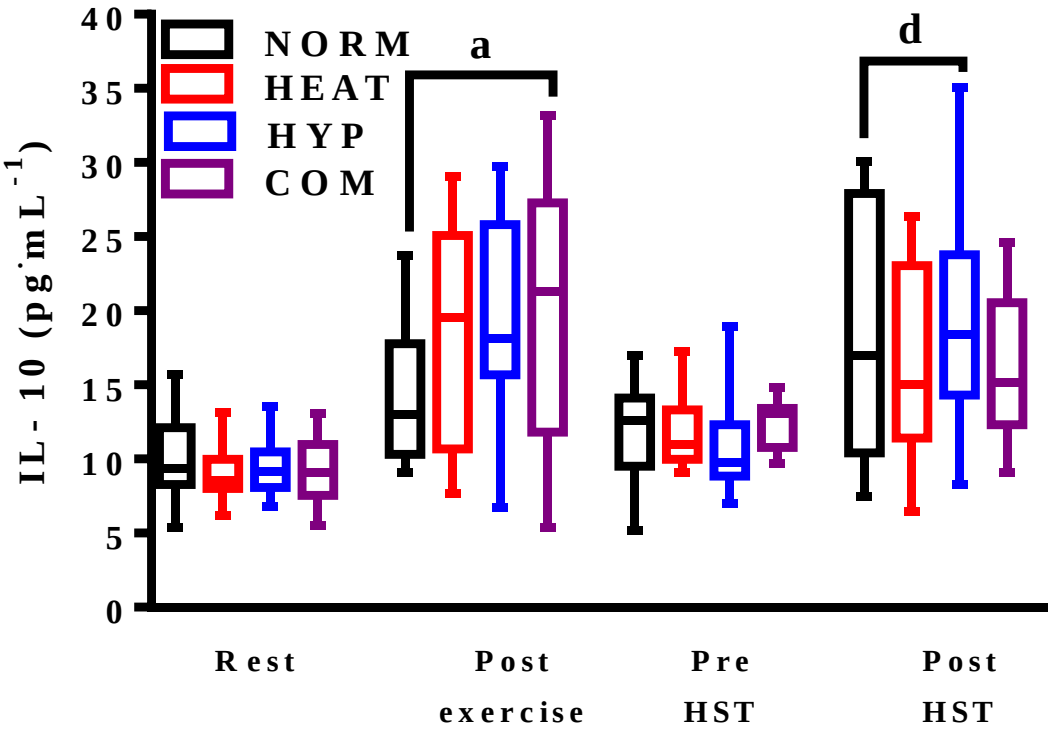

Figure 4.8. Plasma IL-10 at rest, after exercise in each condition, 24 hours after exercise and following the HST IL-10 increased post exercise in each condition (*n* = 12). Letters represent significant differences between means ($p < 0.05$). a = difference from baseline ($P < 0.05$), d = difference from pre HST to post HST. Data show the 1st, 2nd (median) and 3rd interquartile range (coloured boxes) and error bars represent the range from highest to lowest data point achieved time in each condition.

4. 3. 1. 5. Plasma pro/anti-inflammatory cytokines

Resting TNF-α, IL-10 and IL-6 did not vary between conditions (Figure 4.5; $P > 0.05$). Plasma TNF-α was elevated after exercise (main effect for time; $F = 3.556$, $P = 0.025$) and did not vary between conditions (main effect for condition $F = 0.938$, $P = 0.433$). Plasma IL-6 was significantly increased as a result of exercise in all trials. HEAT, HYP and COM each produced greater elevations in IL-6 compared to NORM, with post exercise concentrations in

IL-6 higher in HEAT and COM compared to HYP (condition x time interaction; F = 56.04 P < 0.001). Plasma IL-10 increased post exercise in all conditions, with the magnitude of increase being greater following exercise in HEAT, HYP and COM compared to NORM (condition x time interaction; F = 4.479, P = < 0.001).

4. 3. 2 Post 24 hour HST responses

Exercise times for the HST were not different between trials. Only 1 participant was unable to complete the full 60 minutes exercise in each trial. Participant 6 completed 46, 48, and 46 minutes of exercise in HST_{NORM}, HST_{HEAT} and HST_{HYP} respectively. Results were therefore analyzed using pairwise comparisons up to 40 minutes, with the final values obtained at the end of each test also included in the analysis.

4. 3. 2. 1 Cardiorespiratory responses to the HST

The previous day's exposure had no effect on any resting variable (P > 0.05). Exercising HR had a tendency to be lower in HST_{COM} and HST_{HEAT} compared to HST_{NORM}. HR was ≈ 4 and 5 beats•min^{-1} lower at the end of exercise in HST_{HEAT} and HST_{COM} compared to HST_{NORM} and HST_{HYP} respectively (main effect for condition; F = 2.405, P = 0.08; Figure 4.9). Throughout exercise HR was ≈ 2% lower in HST_{HEAT}, and ≈ 3% during HST_{COM} whereas no reduction in HR in relation to HST_{NORM} was found in HST_{HYP} (main effect for condition; F = 3.351, P = 0.05; Figure 4. 9). SpO_2 during rest, exercise and at the end of exercise also did not vary between conditions (P > 0.05). $\dot{V}_E$ (BTPS and STPD), $\dot{V}O_2$, $\dot{V}CO_2$, and RER did not vary between trials during exercise or at completion of the HST (P > 0.05). Table 4.4 shows the end point data for each HST.

Table 4. 4. Mean ± SD cardiovascular, metabolic, thermoregulatory and subjective data upon termination of each hypoxic stress test (n = 12).

	HST_{NORM}	HST_{HEAT}	HST_{HYP}	HST_{COM}
Cardiovascular				
HR (beats•min^{-1})	150 ±16	146 ± 16	150 ±13	145 ±17
SpO_2 (%)	83 ± 2	82 ± 2	83 ± 2	82 ±2
$\dot{Q}$ (L•min^{-1})	16.9 ± 2.7	15.9 ± 2.6	16.1 ± 2.6	16.0 ± 2.8
Stroke volume (mL•beat^{-1})	117 ± 31	110 ± 22	111 ± 27	113 ± 26
a-$\bar{v}O_2$ difference (mL•L^{-1})	11.1 ± 0.9	11.1 ± 1.4	11.1 ± 1.2	11.0 ± 0.9
Plasma volume (% change)	1.8 ± 7.6	8.3 ± 7.7	2.1 ± 4.7	3.1 ± 6.4
Metabolic				
$\dot{V}O_2$ (L•min^{-1})	1.89 ± 0.41	1.77 ± 0.41	1.79 ± 0.32	1.75 ± 0.42
$\dot{V}CO_2$ (L•min^{-1})	1.85 ± 0.33	1.70 ± 0.32	1.73 ± 0.31	1.66 ± 0.3
RER	0.99 ± 0.10	0.97 ± 0.10	0.98 ± 0.10	0.95 ± 0.10
$\dot{V}_E$ STPD (L•min^{-1})	50.3 ± 11.3	46.0 ± 13.1	47.3 ± 8.8	42.1 ± 8.4
$\dot{V}_E$ BTPS (L•min^{-1})	59.6 ± 9.7	56.0 ± 16.0	57.9 ± 11.0	51.4 ± 10.0
Thermoregulatory				
T_{core} (°C)	37.9 ± 0.2	37.7 ± 0.5	37.9 ± 0.2	37.7 ± 0.3
T_{skin} (°C)	32.1 ± 1.5	32.1 ± 1.1	32.1 ± 1.5	32.5 ±1.8
T_{body}(°C)	36.7 ± 0.3	36.5 ± 0.4	36.8 ± 0.3	36.7 ± 0.5
PSI (AU)	5.1 ± 0.8	4.6 ± 1.4	5.0 ± 0.9	4.6 ± 0.9
Perceptual				
RPE (A.U)	15 ± 3	15 ± 2	14 ± 2	14 ± 1
TS (A.U)	6 ± 1	5 ± 1	5 ± 1	5 ± 1

4.3.2.2 Thermoregulatory responses to the HST

Resting core, skin, and body temperature were unaffected by the previous days exposure ($P > 0.05$). During exercise core temperature had a tendency to be lower in the HST_{HEAT} and HST_{COM} compared to HST_{NORM} and HST_{HYP} (main effect for condition; $F = 2.872$, $P = 0.051$; Figure 4.10). However mean skin and body temperature were not different during the exercise period or upon termination of exercise between trials ($P > 0.05$; Table 4.4). Plasma volume was significantly increased from day 1 to day 2 in all trials (main effect for time; $F = 13.289$, $P = 0.004$), though no differences were found between experimental conditions ($F =$

1.494, P = 0.234). Post exercise changes in plasma volume did not vary between the experimental conditions (F = 0.945, P = 0.430; Table 4.4).

4.3.2.3. Physiological strain index

Physiological strain had a tendency to be lower throughout HST_{HEAT} and HST_{COM} compared with HST_{NORM} and HST_{HYP} (main effect for condition; F = 2.539, P = 0.073; Figure 4.11). When compared to the HST_{NORM}, PSI was ≈ 15% lower throughout HST_{HEAT} and ≈ 11% lower throughout HST_{COM}, with PSI upon the end of exercise being 10% and 11% lower in these trials compared to HST_{NORM}. HST_{HYP} had nominal effect on PSI 24 hours later (Figure 4.11). PSI during the HST_{HEAT} and HST_{COM} trials was ≈ 10% lower than during HST_{HYP} trial. This observation was not statistically significant (F = 2.401, P = 0.116).

4.3.2.4 Ratings of perceived exertion and thermal sensation

Ratings of perceived exertion were not affected by the preceding environmental stressor (main effect for condition; F = 0.98, P = 0.41). Thermal sensation was found to be higher at rest during HST_{HEAT} (F = 9.131, P = 0.01) and HST_{HYP} (F = 4.661, P = 0.05), but no different in HST_{COM} (F = 1.941, P = 0.191) compared to HST_{NORM}. At the end of exercise TS was lower in all experimental conditions compared to HST_{NORM} (P < 0.05; Table 4.4).

4.3.2.5 Monocyte HSP72 responses to HST

mHSP72 had returned to near baseline values in HST_{NORM} (97 ± 9%) but were significantly elevated from baseline in HST_{HEAT} (130 ± 19%), HST_{HYP} (118 ± 17%), and HST_{COM} (131 ± 19%; P < 0.01). mHSP72 was significantly increased from HST pre to HST post in HST_{NORM} (118 ± 12%; P < 0.05). This did not occur in any other experimental condition (P > 0.05).

4.3.2.5. Plasma pro/anti-inflammatory cytokine responses to the HST

TNF-α remained unchanged 24 hours after the initial environmental exposure in all conditions ($P > 0.05$). Plasma IL-10 had returned to near resting values prior to each of the HST ($P > 0.05$). Post HST, IL-10 was increased in relation to day 1 baseline values in each condition except HST_{COM} ($P > 0.05$). In relation to the pre HST sample, IL-10 was elevated post exercise in HST_{HYP} ($P < 0.05$). IL-6 remained elevated 24 hours later in all trials compared with initial baseline values ($P < 0.01$), with pre HST_{HEAT}, HST_{HYP} and HST_{COM} values all higher than pre HST_{NORM} ($P < 0.01$). Pre-HST_{HEAT} and Pre-HST_{COM} were higher than pre-HST_{HYP} ($P < 0.01$). Post HST, IL-6 was increased in all trials ($P < 0.01$), with the greatest increase occurring in HST_{HEAT}, whereby IL-6 concentrations were higher than all other post HST values ($P < 0.01$). In comparison with post exercise values following NORM, HEAT, HYP and COM on day 1, IL-6 was higher in HST_{NORM} ($P < 0.01$), reduced in HEAT and COM ($P < 0.01$), and not significantly different between post HYP and post HST_{HYP} values ($P > 0.05$).

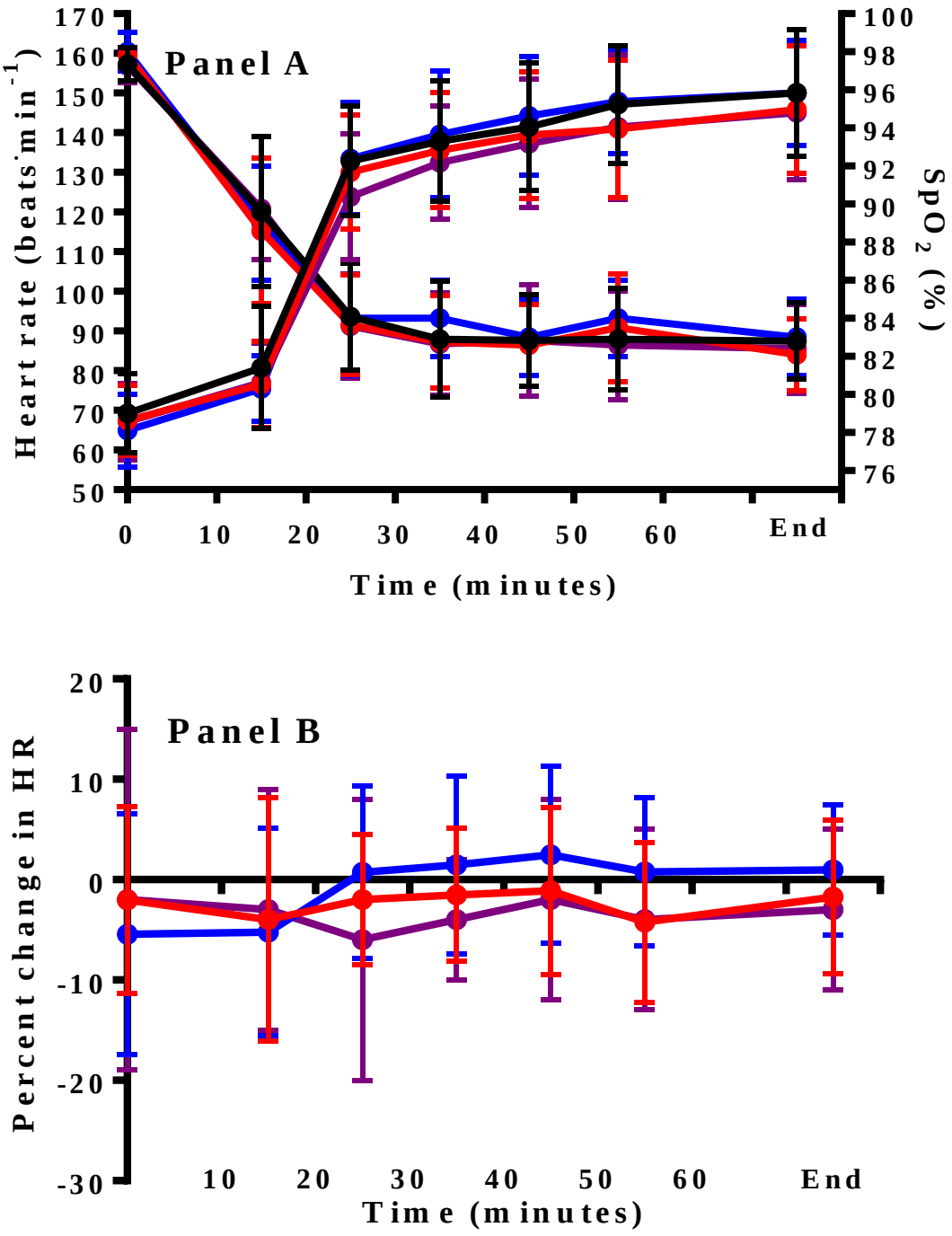

Figure 4.9. Mean ± SD physiological strain index (PSI) during each HST (panel A) and percentage difference in PSI during each HST compared to HST_{NORM} (panel B) (n = 12).

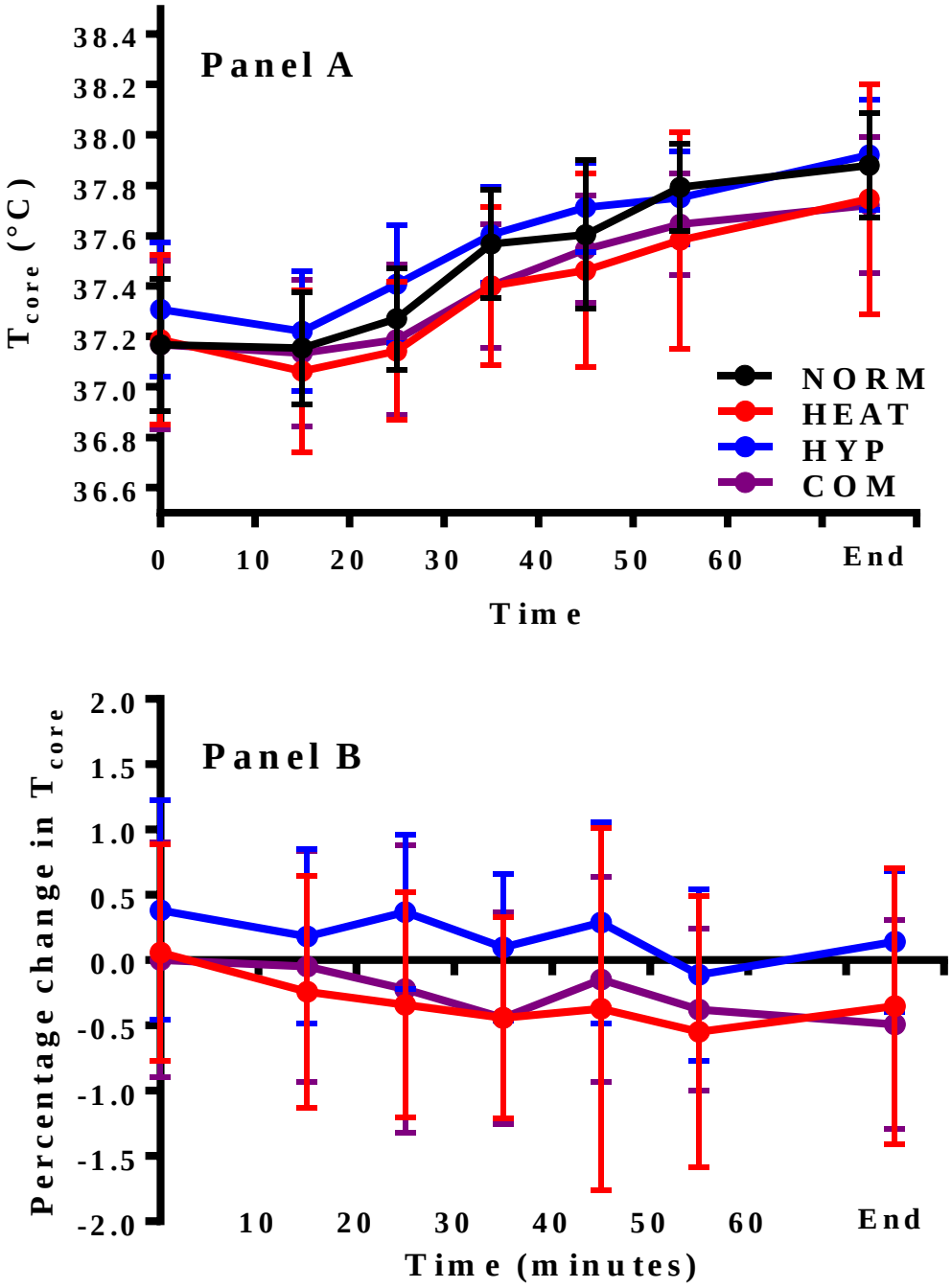

Figure 4. 10. Mean ± SD core temperature during each HST (panel A) and percentage difference in core temperature during each HST compared to HST_{NORM} (Panel B).

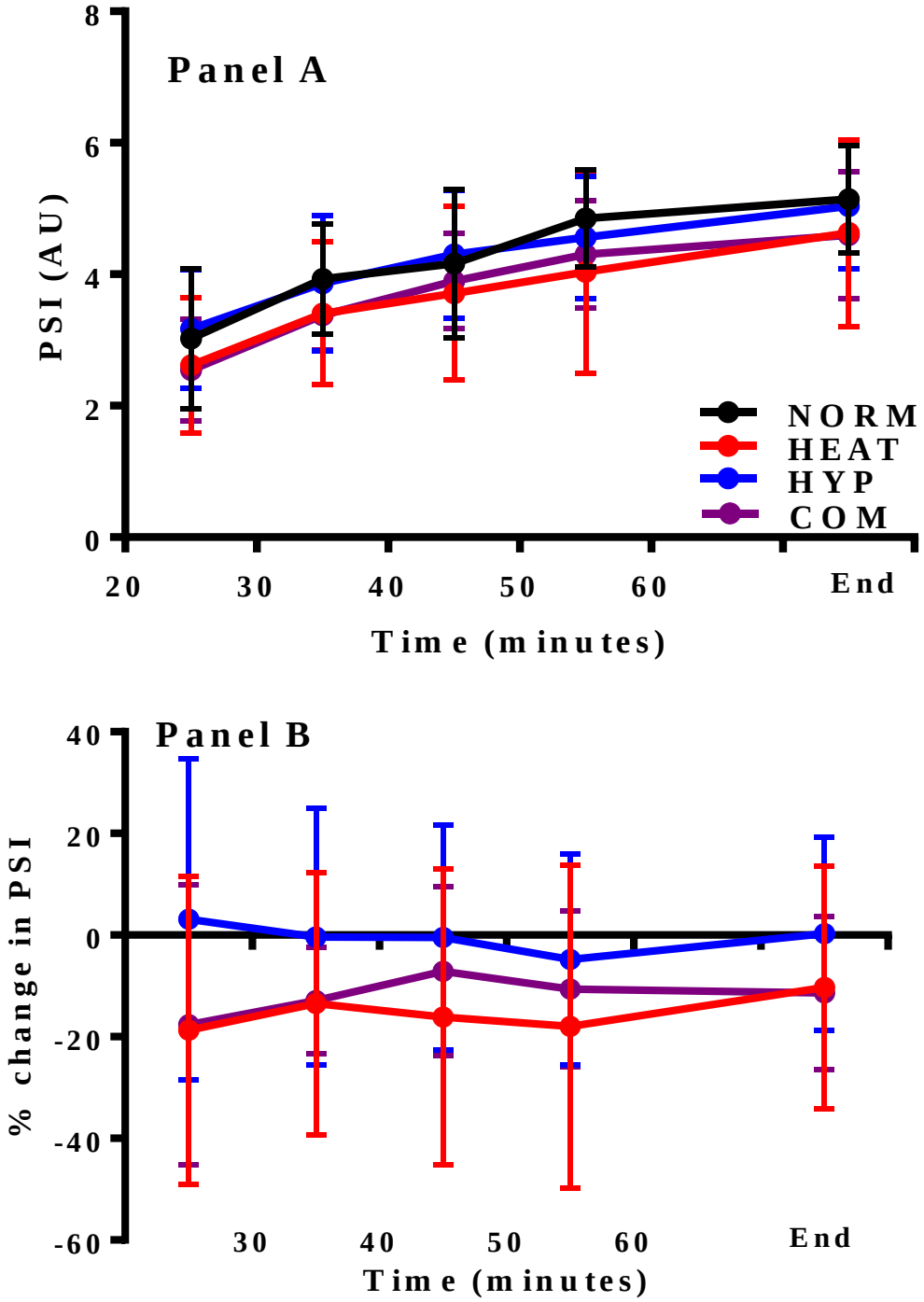

Figure 4. 11. Mean ± SD physiological strain index (PSI) during each HST (panel A) and percentage difference in PSI during each HST compared to HST_{NORM} (panel B).

4.4 Discussion

The major findings of this study were that at the levels used to expose participants within this investigation HEAT induced a greater magnitude of physiological and cellular strain than HYP. The combination of HEAT and HYP induced greater physiological strain than HEAT or HYP alone, supporting the first experimental hypothesis, although post exercise mHSP72 expression was similar between HEAT and COM. A prior acute exposure to HEAT or COM increased basal mHSP72, reduced exercising HR during fixed work hypoxic exercise 24 hours later and attenuated the post exercise mHSP72 expression, supporting the second experimental hypothesis. A prior acute exercise bout in hypoxia did not affect hypoxic tolerance 24 hours later. On balance the results suggest that the perturbations to homeostasis induced during an acute heat exposure (40°C) are greater than those resulting from hypoxia (3000m). Furthermore, the increased level of systemic strain provided by HEAT had a greater impact on subsequent fixed work exercise in hypoxia, whether hypoxia was a feature of the initial stressor or not.

4.4.1 Increased physiological strain enhances the preconditioning response

It is well documented in animal models that a prior preconditioning exposure to a stressor, such as heat, or ischemia, can improve tolerance and/or survival when later exposed to a different stressor (Horowitz 2007). It has been suggested it is the level of strain, and not solely a stress specific response, which drives adaptive processes (Cotter 2013). It is this generalized response to disruptions in homeostasis that may facilitate any preconditioning or cross acclimation response. It is likely that for a cross-acclimation effect to be present, the variant stressors must share some common acute and adaptive responses (Horowitz 2007). For example, the redistribution of blood flow to the skin during a period of heat stress renders some tissues ischemic. This localized ischemia may also act as a stimulus for induction of

HSP72, and also prime the system for later ischemic/hypoxic insult. Of note is the observation that splanchnic tissues undergo ischemia during body heating (Hall et al. 1999), and that this tissue has been strongly linked with the release of HSP72 into the circulation (Febbraio et al. 2002). It is possible that some of these localized ischemic responses to whole body heating activate similar cellular and systemic responses which are seen during whole body hypoxia, and this may play a role in preconditioning and cross acclimation between heat and hypoxic stressors.

An interesting observation in the present study was that HEAT (40°C) and HYP ($F_IO_2 \approx 0.14$) produced a similar level of cardiovascular strain during the initial 40 minutes of exercise (Figure 4.3, 140 ± 8 beats•min^{-1} in HEAT compared to 138 ± 7 beats•min^{-1} in HYP), and each induced post exercise upregulation of mHSP72 and IL-6 (Figure 4.5, Figure 4.7), representing both physiological and cellular common responses. However, the magnitude of the cellular stress response was greater in both HEAT and COM and could be due to the greater physiological strain experienced in these conditions (Figure 4.4). After the initial 40 minutes of exercise in HEAT, T_{core} maintained its rate of rise during HEAT (0.03°C·minute^{-1}), whereas it plateaued in both NORM and HYP trials from 20 minutes onwards (Figure 4.4). The significantly higher heart rate (approx. 12 beats•min^{-1}) upon termination of exercise in the HEAT compared to HYP was probably due to a reduced ventricular filling time and end diastolic function (EDV), mediated by central (ANS) or peripheral factors, such as the direct effect of heat on the SA node, increasing the rate of cardiac contraction (Rubin 1998). As hypothesized, exercise in COM further augmented HR during rest and submaximal exercise (Table 4.1, Figure 4.3). During the initial 40 minutes of exercise in COM HR was ~10beats•min^{-1} higher compared to HEAT and HYP, (Figure 4.3) and as a result PSI was increased throughout exercise in COM compared to both HEAT and HYP (Figure 4.4).

Although the COM exercise condition was, on average, ~6 minutes shorter than HEAT, final T_{core} and PSI were similar (Figure 4.4), indicating a similar magnitude of overall physiological strain was incurred in these conditions. It is possible to infer that heat *per se* induces the greatest degree of overall cellular strain per unit time due to the increased post exercise mHSP72 and IL-6 seen in both HEAT and COM compared to HYP (Figure 4.5, 4.7). The HSR, and expression of heat shock factor-1 (HSF-1) was activated in all environmental conditions as evidenced by post exercise mHSP72 expression and the HSF-1 mediated IL-10 increases (Figure 4.8).

Pre HST mHSP72 values for HEAT HYP and COM were similar to, or greater than those observed post HST_{NORM} (122 ± 13%). Accordingly, a blunted post exercise HSR was subsequently seen post HST_{HEAT}, HST_{HYP} and $HST_{COM.}$ Previous research has shown that the HSR in monocytes is directly proportional to the amount of HSP72 present in the cell (Vince at al., 2010). Conceptually, the monocyte would not require further *de-novo* synthesis of mHSP72 as the elevated basal concentrations would allow the cell to cope with HST induced alterations in cellular homeostasis. IL-10 was, in comparison to the other conditions, unaffected by HST_{COM}. The HSR and activation of HSF-1 are implicated in anti-inflammatory responses to stressors (Xiao et al., 2006), thus the increased cellular tolerance conveyed as a result of COM may have affected cytokine signal transduction and gene expression via an inhibition of NF-KB thus preventing expression of the pro inflammatory mediators such as IL-6 (Amorim and Mosely, 2010). These results indicate that heat per se may induce HSR/HSF-1 mediated anti-inflammatory effects during later hypoxic exercise. Further study should investigate the relationship between HSF-1, HSP72 and both pro and anti-inflammatory cytokines.

Physiologically, a prior exposure to either HEAT or COM led to modest reductions in exercising HR and T_{core}, and therefore PSI during their respective HST (Figure 4.9; 4.10; 4.11). In contrast, a prior exposure to hypoxia in the preceding 24 hours appeared to have no effect on reducing exercising HR as HR values observed during HST_{HYP} were similar to those in HST_{NORM} (Figure 4.9). Physiological strain during the HST was also lower following a prior exposure to HEAT and COM in 9 and 10 participants respectively, whereas PSI during exposure to HYP was only reduced in comparison to NORM in 6 out of the 12 participants. This indicates that a prior exposure to a heat stressor improves tolerance to submaximal exercise in hypoxia. Mechanistically, it is possible that an increased plasma volume effect following each heated trial lead to the reduction in HR, however, no significant statistical change in plasma volume was observed between the 4 trials, however each trial resulted in a slight increase in PV 24 hours later. It is possible that the duration of exercise impacted on the degree of plasma volume expansion experienced, although in this instance PV expansion was not related to exercise time. Despite this, it does seem the most plausible explanation for the reduction in exercising HR in HST_{HEAT} and HST_{COM}. On average participants had expanded plasma volumes 24 hours after HEAT and COM, but not all participants displayed this characteristic. The role physiological strain *per se* has on adaption and subsequent hypoxic tolerance could be further investigated by utilizing a level of hypoxia that induces a greater level of physiological strain than both the levels of HYP and HEAT applied within the present study.

4.4.2 Exercise in the heat offers a more efficient acute training stimulus than hypoxia

The popularity of normobaric altitude training amongst athletes has grown in recent years, despite remaining questions regarding efficacy in improving sea level performance and performance in hypobaric conditions (Beidleman et al. 2014, Muza 2007). The results from

the current investigation indicate that during an acute fixed work exercise bout heat presents the greater physiological and cellular training stimulus compared to normobaric hypoxia at the levels studied.

For example, the acute inflammatory response has been shown to play an important role in the response and adaptation to training (Petersen and Pedersen 2005), with IL-6 shown to mediate the metabolic changes during exercise (Pedersen and Febbraio 2008). These results indicate that training at the same absolute workload under conditions of heat stress provide a more potent training stimulus than when performing the same work bout at ~3000m asl. It is also inferred that HEAT induced a greater level of physiological strain at a lower relative workload than achieved in acute hypoxia.

As maximal oxygen consumption decreases with increasing altitude (Calbet et al. 2003, Wagner 2000) and increasing ambient temperatures (Lee et al. 2013, Lorenzo et al. 2010), absolute workloads under these conditions will be relatively more intense than when performed at sea level. The degree of hypoxia studied in this present investigation has been shown to reduce maximal aerobic capacity to a greater extent than exposure to 40°C heat when compared to values obtained during sea level (HYP = 35 ± 22%, HEAT = 13 ± 11%;) (Lee et al. 2013). As mean exercise intensity did not vary during the HEAT, HYP and COM trials, it suggests that for the lower relative workload, heat is the greater inducer of IL-6 and mHSP72, and thus represents a greater level of systemic strain than the level of hypoxia studied. These results are aligned with that of Lundby et al. (Lundby and Steensberg 2004) who reported cycling exercise performed at the same absolute work intensity (50% of normoxic $\dot{V}O_2$ max) at an altitude of 4100m elicited a three-fold increase in IL-6 compared to that seen at sea level, providing further evidence that exercise intensity augments the IL-6 response (Lundby and Steensberg 2004, Mazzeo et al. 2001). Similarly, prolonged cycling for

90 minutes in the heat at 70% $\dot{V}O_2$ max induced a 4-fold increase in IL-6 compared to a normothermic control condition (Starkie et al. 2005). Heat may offer the greatest practical benefit as an adjunct to training as it elicits a greater physiological and cellular response at a lower, environment specific, workload and for the same level of perceived exertion as experienced in hypoxia (Table 4.3). Individuals using this approach would also have the option of working at higher work intensities than are possible under hypoxic conditions due to the increased reductions in aerobic capacity experienced in hypoxia. Heat acclimation regimens that elevate plasma volume have been shown to improve physical performance ($\dot{V}O_2$ peak and time trial performance) at sea level in well-trained participants (Lorenzo et al. 2011, Lorenzo et al. 2010) and cognitive function during acute hypoxia (Heled et al. 2012). Thus those looking for an adjunct to training may consider the potential benefits of acute and repeated heat training sessions over the more commonly applied altitude model of training.

4.4.3 Exercise tolerance to fixed work exercise in heat and hypoxia is highly variable

Within and between participant exercise capacity was varied between the 4 environmental stressor conditions (Figure 4.2). These results confirm data that suggest that aerobic capacity, to some extent, affects exercise tolerance to both heat (Cheung and McLellan 1998, Selkirk and McLellan 2001) and hypoxia, with those individuals more adapted to endurance exercise better able to regulate their responses to these environmental stressors. These differences become more apparent when participants were separated into trained (> 50ml•kg^{-1}•min^{-1}, 55.8 ± 5.5ml•kg^{-1}•min^{-1}, n = 6) and untrained (< 40ml•kg^{-1}•min^{-1}, 38 ± 2.4ml•kg^{-1}•min^{-1}, n = 6) groups. It is well established that endurance trained athletes behave physiologically as though already adapted to heat stress (Garrett et al. 2009) via an increased heat loss capacity and decreased rectal temperature (Armstrong and Pandolf 1988). This is illustrated by the slower adaptation to heat seen in those with lower levels of aerobic fitness, compared to their trained

counterparts [24]. Heat acclimation has been shown to increase sweat rate and decrease rectal temperature without effecting performance in a trained group of similar aerobic fitness seen in the current investigation (>55mL•kg^{-1}•min^{-1}), whereas in the untrained group, sweat rate was increased with no changes seen in rectal temperature or exercise performance. The authors concluded that aerobic fitness resulted in significant improvements in exercise heat tolerance, regardless of acclimation status (Cheung and McLellan 1998). Thus the variation in performance seen in this present study may be, in part, related to the training status of participants.

Motivation may have played a factor in the termination of trials, as early termination was not always coincident with a maximal RPE of 20. RPE increased linearly with time in all conditions, however RPE following the initial 10 minutes of exercise was higher in the 3 environmental stress conditions compared to NORM. From a perceptual perspective one could speculate that heat is a more habitual stressor than hypoxia, thus natural tolerance, and understanding of the physical sensations involved when working under an imposed heat load would be greater than that experienced in hypoxia. None of the participants used in this study had ever been to an altitude of >2500m, whereas all had at some stage experienced high ambient temperatures as part of a seasonal variation in climate. Thus the novel sensations experienced during the hypoxic sessions may have, in part, contributed to cessation of these trials. The increases in skin temperature during the hyperthermic trials, and the reductions in arterial oxygen saturation during the hypoxic conditions may have increased the set point for the rate of RPE increase, and partially explain some of the differences in exercise capacity observed (Crewe, Tucker and Noakes 2008).

4.4.4 Experimental considerations

The exercise duration during the preconditioning exercise bout on day one of each trial may have impacted upon any reductions in HR and T_{core}. Therefore future studies employing a similar model are advised to control for exercise duration during the initial bout, ensuring all participants are exposed to the preconditioning stressor for the same length of time. Utilizing fitter participants in future studies may allow for an equal preconditioning dose to be administered across each environmental condition. Alternatively reducing the exercise intensity may also allow for a consistent exercise dose. This would enable more robust conclusions to be made about the effects of a prior heating exposure on hypoxic tolerance. However this approach would have compromised the performance capacity aspect during the first stage of this study.

It was important that baseline mHSP72 on the first day of each 2 day trial period did not vary between conditions, as the rate of appearance of HSP72 post heat stress has been shown to be relative to the monocytes basal HSP72 content (Vince et al. 2010). The 7-day washout from the end of a HST to the beginning of the next trial allowed resting mHSP72 to return to baseline values. It was not possible to examine the time course of this response, nor was the gene expression profile of HSP72 assessed as part of the current investigation. Morton et al. (Morton et al. 2006) reported that intramuscular HSP72 peaked at 72 hours after a non-damaging running protocol, with values still elevated 7 days after the initial exercise bout. It is possible that the recruitment of a larger muscle mass coupled with eccentric muscle activity may prolong this post exercise elevation in HSP family members compared to the cycling exercise used in the present study. Khassef et al. (Khassaf et al. 2001) utilized a one legged cycling protocol to elevate intramuscular HSP72. They reported a large inter-individual response to the exercise bout, and HSP72 values remaining elevated 3-6 days after exercise.

It is therefore possible that each prior trial had a residual effect on intramuscular HSP72 levels that were not reflected in the intracellular samples, collected from the systemic circulation, as part of this investigation. Each experimental block was randomized and completed the trials in different orders thereby minimizing the potential confounding effects described above. However the time-course of the intramuscular HSP72 response, and how this correlates with systemic intracellular HSP72 warrants further investigation.

4.5 Conclusion

Although exploratory in nature, the results from this study reveal that the levels of heat and hypoxia used produce similar degrees of cardiovascular strain for approximately 40 minutes of exercise at a work rate of 50% $\dot{V}O_2$ peak. It is anticipated the novel findings of this study will provide a starting point for those interested in investigating different combinations of heat and hypoxia, and how these impact upon physical performance. As expected, when heat and hypoxia are combined, acute physiological and cellular stress responses are augmented. However, the level of heat used in this present investigation appears to produce a greater physiological stress response 24 hours later compared to the level of hypoxia used, with the combination of two stressors not eliciting greater effects than the use of heat alone. The finding that heat stress *per se* appears to elicit a greater adaptive stimulus than the level of hypoxia studied could have several practical implications. For example, periods of heat training could be implemented into an athlete's training schedule, or be used as an efficient and cost effective means of preparing individuals (such as military personal) for rapid redeployment from areas of heat to areas of altitude. Future mechanistic research into short term, whole body preconditioning between heat and hypoxia should control for both duration of the initial exposure, and degree of hyperthermia induced. The effects of a prior,

preconditioning period of whole body, or localized muscle heating on exercise tolerance and performance is also a suggested area for future research.

The preconditioning aspect of the present investigation examined the second window of protection (SWOP) phase of the cross-acclimatory process (i.e. exposure to a stressor 24-48 hours upon removal from the initial stressor). The next step along on the adaptive continuum pertains to short term heat acclimation (STHA), an area that has received greater research interest in human acclimation models (Marshal et al., 2006, 2007; Garnett et al., 2008, 2011). Human sudomotor, hemodynamic and cardiovascular adaptations appear to be initiated with the first few days (2 – 3 days, Marshall et al., 2006, 2007; 3-5 days, Garrett et al., 2011) of repeated daily heat exposures. To date no in-vivo human research has examined whether the initial 3-5 days of a heat acclimation regimen can provide enhanced tolerance to a secondary stressor. The following chapter aims to address whether STHA can be induced with 3 one hour exercise exposures to heat, and whether STHA can confer tolerance to subsequent hypoxic exercise.

Chapter 5. Human monocyte HSP72 responses to acute hypoxic exercise after 3 days of exercise heat acclimation

This experimental chapter has formed the basis of the publication detailed below:

B. J Lee, RWA Mackenzie, A. Hussain, V. Cox, R. S James & C.D. Thake (2014). The impact of 3 days exercise heat acclimation on monocyte heat shock protein 72 and subsequent cellular tolerance to acute exercise in hypoxia in humans. *Biomedical Research International*, Special Issue on Exercise Physiology, Cognitive Function, and Physiologic Alterations in Extreme Conditions.

5.1 Introduction

Monocyte HSP72 (mHSP72) was shown to increase following acute exercise in both heat and hypoxia (Chapter 4). Heat stress, in isolation, induced a greater mHSP72 compared to hypoxia alone. The combination of both heat and hypoxia did not induce a greater HSP response than heat alone, however the exercise bout was shorter as volitional exhaustion was achieved sooner in this condition. mHSP72 remained elevated 24 hours post exercise, and were attenuated following a hypoxic stress test (HST) in the heated groups. The attenuation of post exercise HSP72 occurred in conjunction with moderate reductions in exercising HR, T_{core}, and physiological strain in these conditions. It is unusual for humans to encounter such a brief period of heat exposure in a real life setting, and most heat acclimation protocols are medium (8-14 days) or long term (> 15 days) in their nature

However it is now accepted that many of the beneficial adaptations to heat stress are cardiovascular and occur rapidly over the initial phase (3-5 days) of acclimation (Garrett et al. 2009). A 3-day heat acclimation protocol has previously been shown to reduce exercising HR and T_{core}, increase sweat rates and basal levels of HSP72 mRNA, thus also possibly

conferring cellular tolerance to heat (Marshall et al. 2007). Shorter-term protocols may be logistically easier to implement and therefore more appropriate for an athlete's training, military operations, or within a clinical environment. It would be of interest to determine whether short-duration (1-hour) heat exposures over repeated, consecutive days would induce further increases in basal mHSP72 and improvement physiological markers of hypoxic tolerance compared to the acute exposure utilized in Chapter 4.

It is possible that the elevated mHSP72 and reduced physiological strain following an acute heat exposure shifts the threshold of strain required for further HSP72 induction following any subsequent heat exposures (Katschinski, 2004). Heat acclimation is known to increase in basal stores of HSP72 (Marshall et all., 2006, McClung et al. 2008). However the expression kinetics of this induction was not accounted for in previous longer duration heat acclimation studies with measures made on day 1 and day 10 (Yamada et al., 2007; McClung et al., 2008). The majority of the increase in HSP72 may have occurred within the initial few days of acclimation, and the reduced physiological strain observed latterly in the acclimation period may fail to induce any further expression changes in HSP72. It is, however, known that mHSP72 increased continuously in an almost linear fashion over 10 days of resting hypoxic exposure (Taylor et al. 2010).

As discussed in Section 2.5.5, circulating HSP72 (eHSP72) has been shown to increase (Marshall et al., 2006) or decrease (Sandstrom et al., 2008; Krefelder et al., 2005) during the initial phase of heat acclimation (3-5 days). However these studies utilized either serum measurements, which have been shown to yield lower basal values and be less sensitive to stress induced change (Whitham and Fortes, 2008) or the low sensitivity ELISA that was available at the time (Enzo lifesciences, kit ADI-EKS-700B). Longer-term acclimation studies have reported either an 110% increase in serum HSP72 (Yamada et al., 2007) or no

change in plasma HSP72 (Magalhaes et al., 2010) levels, with a blunted response to additional heat stress compared to pre-acclimation (Magalhaes et al., 2010). Therefore, it would be of interest to determine eHSP72 responses over the initial early phase of heat acclimation using EDTA plasma samples. Additionally no previous study has reported on the eHSP72 response to acute hypoxic exposure in humans.

The primary aim of this study was to determine whether the initial phase of heat acclimation in humans could induce an increase in basal mHSP72 and precondition against a subsequent bout of acute hypoxia when compared to a normothermic control group. Secondly, characterizing the plasma EDTA eHSP72 response to short term heat acclimation (STHA), and the response of eHSP72 to acute hypoxic exercise. It was hypothesised that 3 days of heat acclimation would increase basal stores of mHSP72 and that the increased availability of mHSP72 would attenuate the response of this cytoprotective protein to a subsequent bout of acute hypoxic exercise.

5.2 Methods

5.2.1 Participant characteristics

Sixteen healthy males (mean ± SD; age 21 ± 2 years, height 1.78 ± 0.10m, mass 76.1 ± 9.0kg, estimated body fat 15.2 ± 5.2%, estimated muscle mass 56.3 ± 6 .5%; peak oxygen uptake ($\dot{V}O_{2\ peak}$) 3.4 ± 0.7 L•min^{-1}) provided signed informed consent prior to participation in this study, which was granted approval by the Coventry University Ethics committee. All participants were physically active, non-smokers with no prior history of cardiorespiratory illness. Caffeine (Lu, Lai and Chan 2008) and alcohol consumption were barred from all meals and beverages for 72 h prior to each laboratory visit. Participants were required to

maintain a food and activity diary, as accurately as possible, for 3 days prior to each experimental visit. They were requested to replicate their food intake and activity levels (habitual exercise only) prior to each experimental visit (Morton et al. 2006). Additionally, participants refrained from all supplementation (i.e., vitamins, ergogenic aids) throughout the study period. Participants were requested to abstain from prolonged thermal exposures (baths, saunas, steam rooms, tanning devices) and vigorous physical activity for seven days prior to the preliminary testing, and throughout the remaining experimental program. Furthermore, subjects who had visited or resided at altitudes in excess of 1000 m (Taylor et al. 2010) or climates with ambient temperatures in excess of 30^{o}C (Sandstrom et al. 2009, Selkirk et al. 2009), or had experienced high pressure environments e.g. hyperbaria (Taylor *et al.*, 2011b), three months prior to study commencement, were excluded during recruitment due to the possible influence of such environments on basal HSP72 expression. Participants were required to fast for 2 hours prior to each trial, and did not eat until the final blood withdrawal. Compliance for all the aforementioned experimental controls was monitored via questionnaires administered before, during and post the extended experimental study period and reported compliance was 100% in all participants.

5.2 Experimental design

Participants reported to the laboratory on six occasions, between the hours of 0800 and 1100 as outlined in Figure 5.1. The first visit involved assessment of preliminary measures of anthropometry, lactate threshold and $\dot{V}O_{2\ peak}$. Participants attended the laboratory at the same time of day, on each laboratory visit, in order to minimize the effects of circadian variation on performance (Winget *et al.*, 1985; Reilly *et al.*, 2007) and the known diurnal variation in mHSP72 (Taylor et al. 2010).

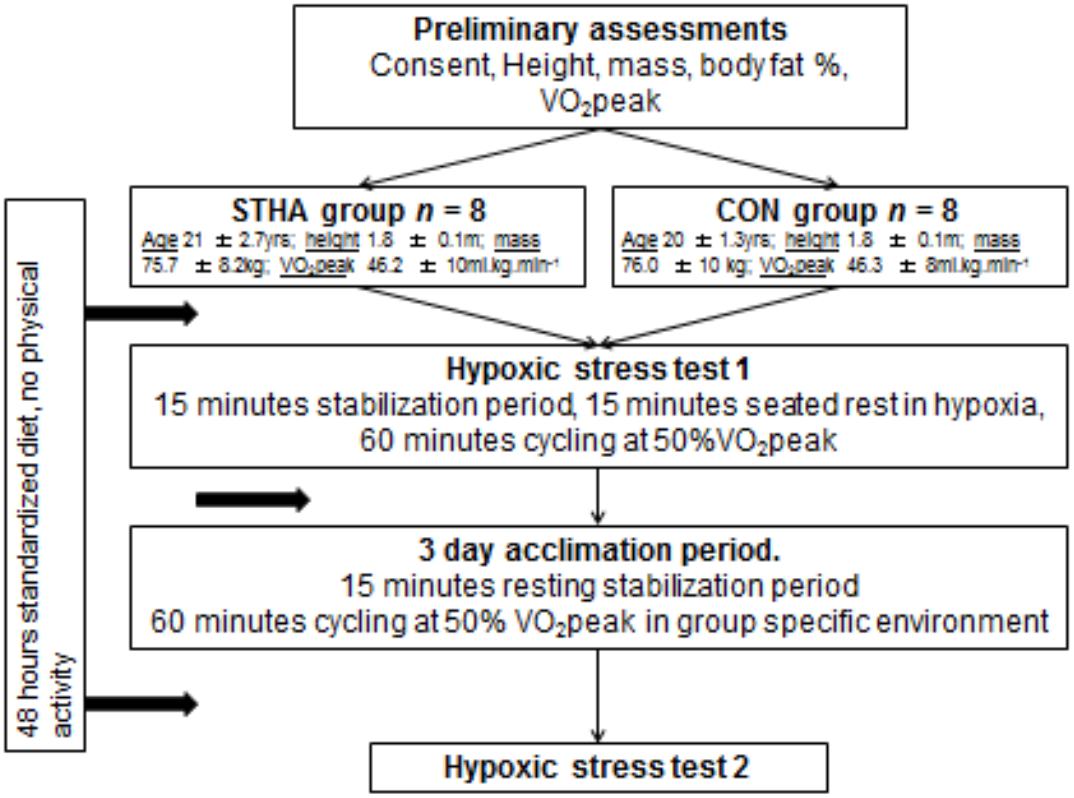

Figure 5.1. Experimental schematic. During the HST measures of HR, SpO_2, T_{core} T_{skin}, PSI, $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}_E$, and RER were collected before and at the end of the rest period, and at 10 minute intervals throughout exercise. After each Douglas bag measurement RPE (Borg, 1970) and thermal sensation (Moran et al.,1996) were noted. During the acclimation period, HR, T_{core}, T_{skin}, PSI, RPE and TS were collected before and after rest, and every 10 minutes throughout the exercise period.

5.2.2.1 Visit 1 – Preliminary testing and hypoxic familiarization

During the initial visit participant's height and body mass were obtained to the nearest 0.1cm or kg respectively in the Frankfurt plane using a Harpenden Stadiometer (Harpenden, UK) and scales (Seca, Bodycare, Southsea, UK). Body fat and muscle was assessed using 4 skin fold sites (tricep, subscapular, medial calf, and thigh) and 2 limb girth measurements

(maximal calf girth and forearm according to the British Association of Sport and Exercise Sciences (BASES) guidelines (Winter et al., 2007). Peak oxygen consumption was determined as describe in Section 3.6.1. Following preliminary testing, the subjects were divided into 2 matched groups based on the baseline measures of $\dot{V}O_2$ and body composition. One group (STHA) completed the heat acclimation protocol at 40°C, whereas the control group (CON) completed the same exercise at 20°C.

5.2.2.2 Visit 2 and 6 - Hypoxic stress test (HST)

At least 5 days after the preliminary visit, participants returned to the laboratory for the baseline HST. This procedure was repeated 48 hours after the final acclimation session. Details of the method are found in Section 3.11. A venous blood sample was collected from an antecubital vein into Potassium coated EDTA vacationers (BD San Jose, California) for the assessment of monocyte HSP72 (mHSP72) and eHSP72 before and after each HST as described in Section 3.12.

5.2.2.3 Visits 3, 4 and 5 - Heat acclimation protocol

Following a 7-day wash out after the initial HST, participants reported to the laboratory to undergo 3 days of heat acclimation (HA) or exercise training. This delay in testing has been shown to be long enough to allow basal levels of mHSP72 to return to their initial values (Section 3.12). Details of the heat acclimation procedures are presented in section 3.9. On HA day 1 and HA day 3 a 7mL venous blood sample was obtained from an antecubital vein into a potassium - EDTA vacutainer (BD, San Jose, California) at rest and upon completion of the exercise bout for the assessment of mHSP72 and eHSP72.

5.2.2.4 Data analysis and statistics

Each data set is presented as mean ± SD in the text, tables and figures. The primary outcome variables of interest in this experiment were the mHSP72 and eHSP72 responses to the HST. A mixed model two-factor ANOVA with repeated measures on the second factor was used to make all group x time comparisons throughout each HST and to assess between and within group differences upon completion of the first and last acclimation day. F values were adjusted for sphericity where appropriate. Main and interaction effects were investigated by Tukey's HSD test. In order to investigate the relationship between pre-exercise and post-exercise induced expression of mHSP72, Pearson correlational analysis was performed. The significance level was set at $P < 0.05$.

5.3 Results

5.3.1 Physiological and perceptual responses to the acclimation period

Participants in both groups were considered hydrated prior to each acclimation session with no differences, between each day, in urine specific gravity or nude body mass upon arrival to the laboratory (Table 5.1). All participants in CON completed the full 60 minutes of cycling on each day. In STHA, 3 participants failed to complete the 60 minutes on day 1 (mean ± SD; 55.5 ± 6.2 mins) compared to 1 on the final day of acclimation (58.5 ± 4.2 mins). Mean and peak HR, T_{core}, T_{skin}, T_{body}, PSI, RPE and TS were significantly higher during all 3 days of acclimation in the STHA group compared to the CON group ($P < 0.01$) (Table 5.2). Plasma volume, mean and peak HR, T_{core}, T_{skin}, T_{body}, PSI, RPE and TS were did not vary from HA1 to HA3 in either group ($P > 0.05$; Table 5.2). Resting plasma volume was unchanged over the course of the acclimation period in the CON ($P < 0.05$) and was increased on day 3 compared to day 1 in the STHA ($P < 0.05$; Table 5.1).

Table 5.1. Mean ± SD resting body mass urine specific gravity prior to each acclimation session. Baseline (day 1) plasma volume was 52.9 ± 2.7 and 54.6 ± 2.9 in the CON (*n* = 8) and STHA (*n* = 8) groups respectively. * Difference from day 1 (P <0.05).

	Day 1	Day 2	Day 3
CON			
USG	1.009 ± 0.004	1.011 ± 0.007	1.008 ± 0.010
Body mass (Kg)	75.8 ± 10.4	75.8 ±10.2	75.9 ± 10.4
Sweat rate	0.55 ± 0.18	0.60 ± 0.33	0.61 ± 0.38
Δ Plasma Volume (%)	-	-1.1 ± 5.1	1.0 ± 4.0
STHA			
USG	1.006 ± 0.005	1.010 ± 0.006	1.009 ± 0.008
Body mass (Kg)	77.4 ± 6.4	77.4 ± 7.1	77.3 ± 6.5
Sweat rate	1.20 ± 0.46	1.42 ± 0.74	1.48 ± 0.51
Δ Plasma Volume (%)	-	1.8 ± 3.9	4.6 ± 5.7*

5.3.2 mHSP72 responses to the acclimation period

Resting mHSP72 was not different between groups on HA1 (P > 0.05). mHSP72 was different between groups following the acclimation intervention (trial x time x group interaction observed F = 17.9, P < 0.001). Post exercise mHSP72 increased immediately following HA1 in the STHA group (158 ± 27%, P < 0.01) but not in the CON group (111 ±

Table 5.2. Peak and mean exercising ± SD physiological and thermoregulatory measures during the 3-day acclimation period for CON (*n* = 8) and STHA (*n* = 8). * indicates a difference between experimental group (P< 0.05).

Measure	Change in NBM (kg)	Peak HR (beats·min^{-1})	Mean HR (beats·min^{-1})	Peak T_{rec} (°C)	Mean T_{rec} (°C)	Peak T_{skin} (°C)	Mean T_{skin} (°C)	Peak T_{body} (°C)	Mean T_{body}(°C)	Peak PSI (A.U)	Mean PSI (A.U)
CON											
Day 1	0.6 ± 0.2	151 ± 21	144 ± 18	38.0 ± 0.2	37.7 ±0.5	33.8 ± 1.3	33.0 ± 1.0	37.2 ± 0.2	36.8 ± 0.2	5.5 ± 1.1	4.6 ± 0.8
Day 2	0.6 ± 0.3	151 ± 23	142 ± 19	38.0 ± 0.2	37.7 ± 0.5	33.7 ± 1.0	32.6 ± 1.3	37.0 ± 0.5	36.7 ± 0.2	5.6 ± 1.3	4.5 ± 0.8
Day 3	0.6 ± 0.4	149 ± 23	143 ± 20	38.1 ± 0.4	37.7 ± 0.2	33.6 ± 0.9	32.8 ± 0.7	37.2 ± 0.3	36.7 ± 0.3	5.7 ± 1.4	4.5 ± 0.9
STHA											
Day 1	1.2 ± 0.5*	180 ± 13*	165 ± 14*	38.8 ± 0.3*	38.1 ± 0.2*	36.6 ± 1.0*	35.9 ± 1.0*	38.3 ± 0.3*	37.6 ± 0.3*	8.3 ± 1.0*	6.0 ± 0.8*
Day 2	1.4 ± 0.5*	176 ± 13*	162 ± 14*	38.7 ± 0.4*	38.0 ± 0.2*	36.6 ± 0.9*	36.1 ± 0.4*	38.3 ± 0.4*	37.6 ± 0.2*	8.1 ± 1.2*	5.9 ± 0.9*
Day 3	1.5 ± 0.5*	173 ± 13*	160 ± 13*	38.5 ± 0.3*	37.8 ± 0.1*	36.2 ± 1.0*	35.7 ± 0.7*	38.1 ± 0.4*	37.4 ± 0.3*	7.8 ± 1.1*	5.7 ± 0.8*

11%, P > 0.05). Resting mHSP72 was elevated from preHA1 to preHA3 in the STHA group (131 ± 23%, P < 0.001) and remained unchanged in the CON group (102 ± 18%, P > 0.05). mHSP72 did not vary from rest following exercise on HA3 in either group (P > 0.05). Post exercise mHSP72 on HA3 was lower compared to the post exercise data on HA1 for the STHA group (P < 0.05) (Figure 5.2). A significant negative correlation (r = - 0.81 P = 0.014) was observed in the STHA group between the pre- exercise expression, and the increases in mHSP72 induced by exercise on heat acclimation day 1 (Figure 5.3 panel A). The relationship was not was not present on the third day of the heat acclimation period (r = - 0.44 P = 0.29; figure 5.3 panel B).

5.3.3 Circulating HSP72 responses to the acclimation period

One participant in the control group was below the detection limit of the assay at each time point during the acclimation period, and was removed from the statistical analysis. Two members of the STHA group were below the detection limit of the assay prior to exercise on day 3 of heat acclimation. These participants were removed from the statistical analysis for the 3-day acclimation period. eHSP72 significantly increased following exercise on day 1 and day 3 of the acclimation period (day 1: increase of 1.06 ± 0.74ng•ml^{-1}; day 3: increase of 1.04 ± 1.06 ng•ml^{-1}; P < 0.001) in the STHA group. Resting eHSP72 was no different (n = 6) on day 3 of HA compared to day 1 (day 1: 1.43 ± 0.15 ng•ml^{-1}; day 3: 1.12 ± 0.54 ng•ml^{-1}; Figure 5.4). eHSP72 remained unchanged from rest following exercise on each day of the 3 day protocol in the CON group (P > 0.05).

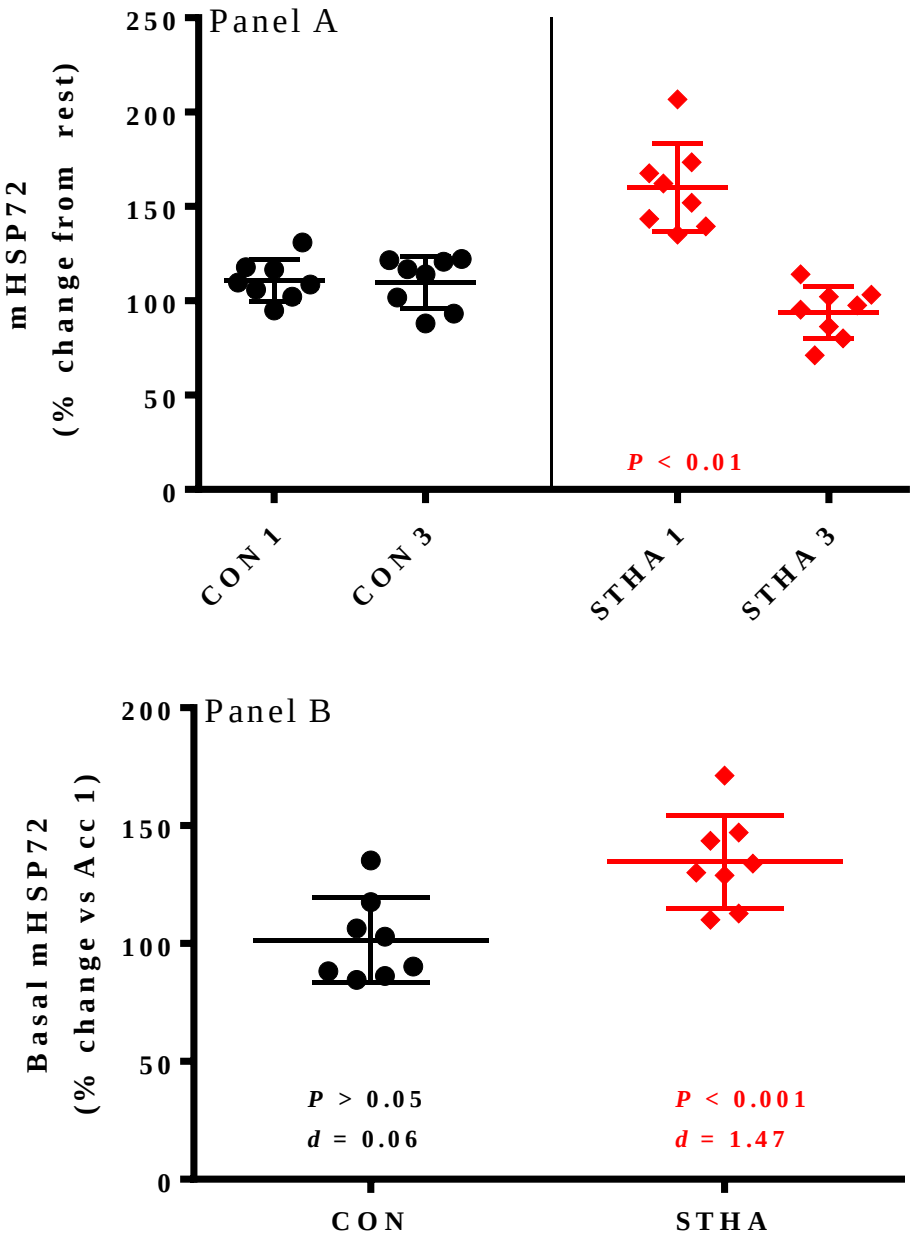

Figure 5.2. Fold change in mHSP72 immediately after exercise on HA1 and HA3 3 of the 3-day heat acclimation/exercise training period (Panel A). Post exercise data is shown relative to the pre-trial value. mHSP72 was increased from baseline on HA1post ($P < 0.01$) but not on HA3post. Basal mHSP72 was elevated on HA3pre compared to HA1pre in the STHA group

(Panel B; $P < 0.001$, $d = 1.47$). Data for each particpant in the CON group (n = 8) and STHA group (n = 8) is shown. The horizontal line and error bars represent the mean and SD.

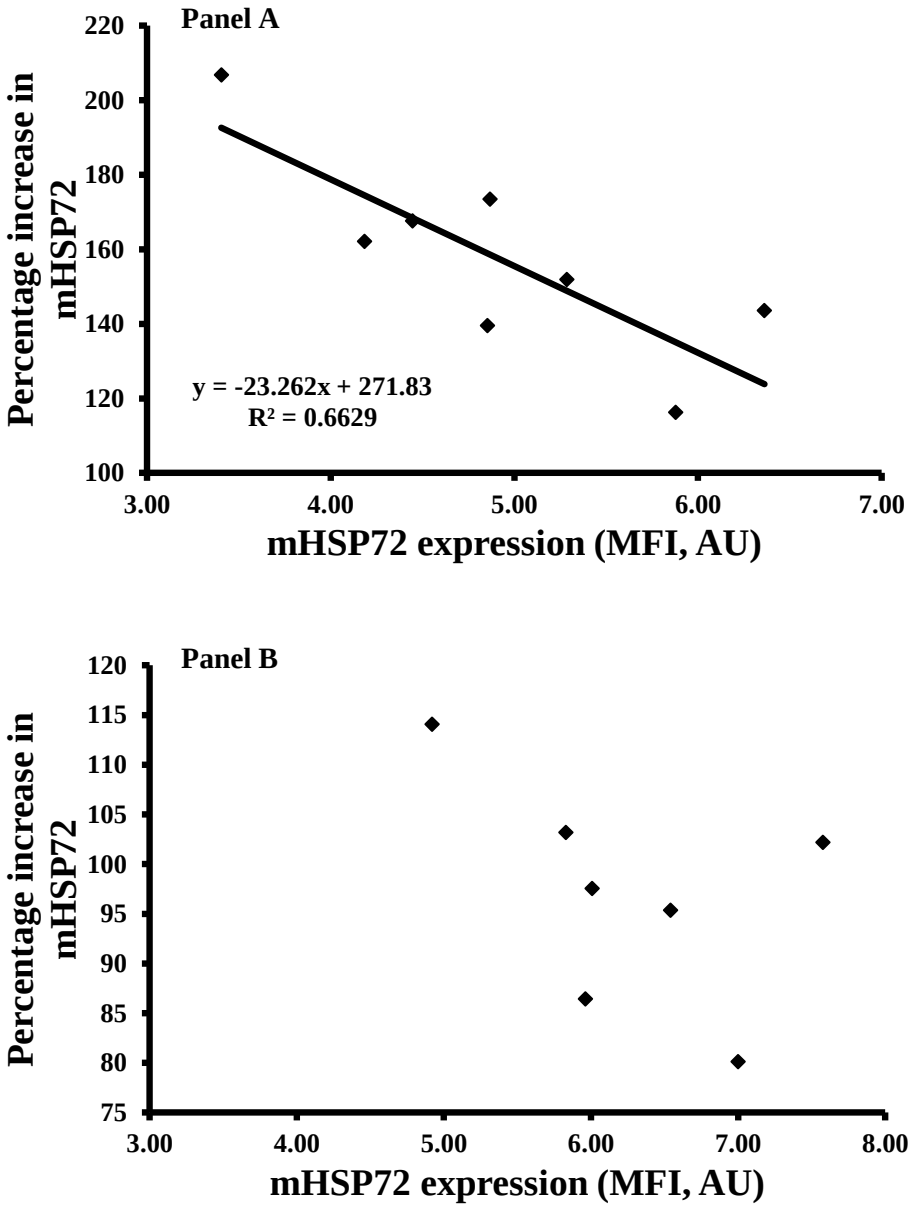

Figure 5.3. A negative relationship exists between basal mHSP72 and post exercise mHSP72 induction before (r = -0.81, P = 0.014; panel A) but not after (r = -0.44, P = 0.29; panel B) in STHA.

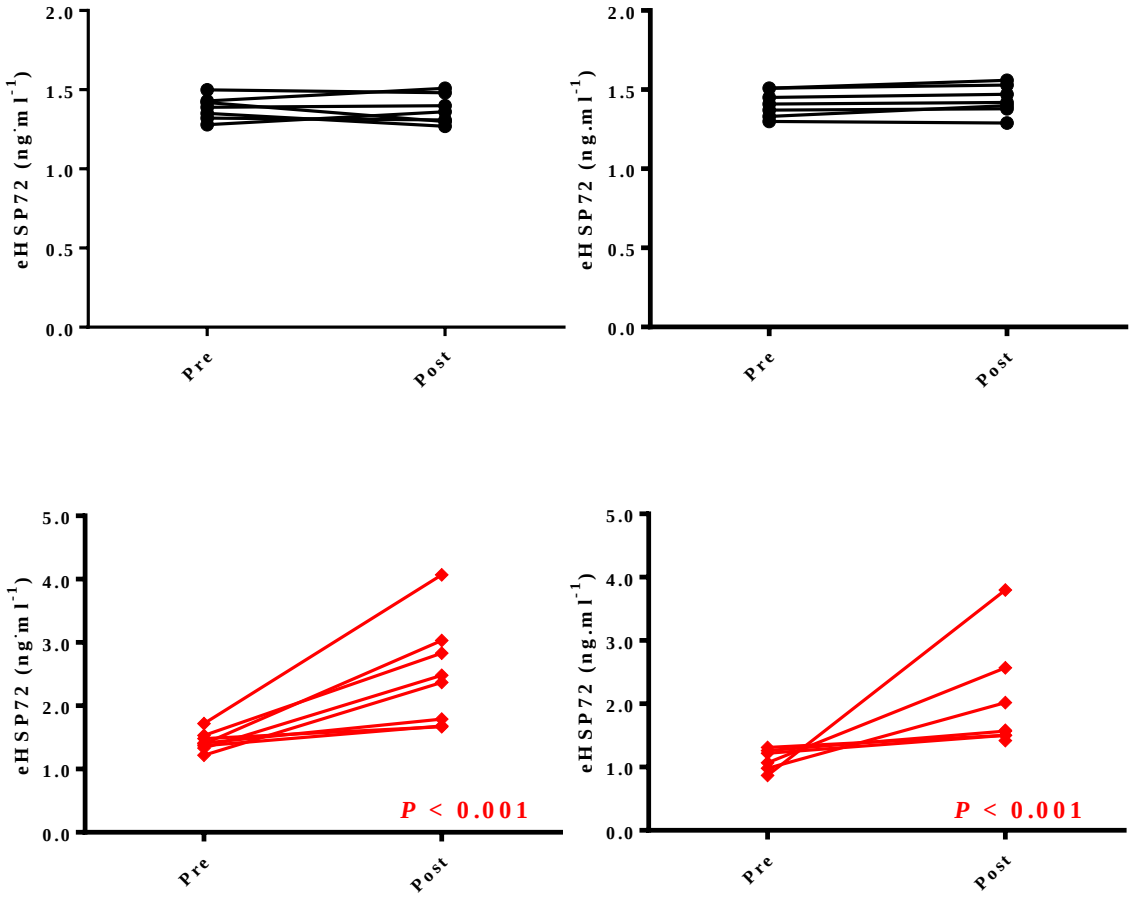

Figure 5.4. The intervention period did not induce any changes in post exercise eHSP72 in CON (n = 8, black lines, Panel A). Post exercise eHSP72 was elevated from rest post exercise on HA1 and HA3 in the STHA group (n = 7, P < 0.001, red lines, Panel B).

5.3.4 Physiological responses to the hypoxic stress test

Heart rate was reduced in HST2 compared to HST1 ($F = 6.99$, $P = 0.019$), however there was no trial x group interaction ($F = 0.628$, $P = 0.441$). HR was lower in HST2 than HST1 between 20 and 60mins for STHA, but only between 20 and 30mins for CON (trial x time interaction, $F = 2.47$, $P = 0.039$). S_PO_2 was higher throughout HST2 compared with HST1 in STHA ($F = 10.4$, $P = 0.006$), and between 20 and 30mins in CON, although no trial x group interaction was observed ($F = 0.15$, $P = 0.70$) (Figure 5.5). HR and SpO_2 data are shown in Figure 5.5. End of exercise data for selected physiological, metabolic and thermoregulatory variables are presented in Table 5.3.

5.3.5 Thermoregulatory responses to the hypoxic stress test

A main effect for trial was found for both core ($F = 4.75$, $P = 0.047$) and skin mean temperature ($F = 10.5$, $P = 0.006$) but not for mean body temperature ($F = 0.485$, $P = 0.497$). Final core temperature during HST1 for the STHA group was 38.1°C (95% C.I 37.8 – 38.5°C) and 38.1°C (95% C.I 37.7 – 38.4) for the CON group. At the end of HST2 peak core temperatures were 37.8°C (95% C.I 37.7 – 38.0°C) and 37.9°C (95% C.I 37.7 – 38.0°C) for the STHA and CON group respectively. Physiological strain was reduced during HST2 compared to HST1 ($F = 8.653$, $P = 0.01$; Table 5.3). Thermoregulatory data are shown in Figure 5.6.

Table 5.3. Peak and mean exercising (± SD) physiological and thermoregulatory measures for the pre (HST1) and post acclimation (HST2) hypoxic stress tests. * indicated a difference between HST1 and HST2 ($P < 0.05$).

Measure	Change in NBM (kg)	Peak HR (beats·min^{-1})	Mean HR (beats·min^{-1})	Peak T_{core} (°C)	Mean T_{core} (°C)	Peak T_{skin} (°C)	Mean T_{skin} (°C)	Peak T_{body} (°C)	Mean T_{body} (°C)	Peak PSI (A.U)	Mean PSI (A.U)
CON											
HST1	0.45 ± 0.3	162 ± 19	157 ± 15	38.1 ± 0.4	37.8 ± 0.4	33.5 ± 1.2	32.6 ± 1.0	37.2 ± 0.4	36.8 ± 0.5	6.0 ± 1.2	5.3 ± 1.2
HST2	0.50 ± 0.2	160 ± 8	154 ± 12	37.9 ± 0.3	37.8 ± 0.3	34.0 ± 1.0	33.4 ± 1.3	37.3 ± 0.1	36.9 ± 0.4	5.8 ± 0.8	4.9 ± 1.1
STHA											
HST1	0.51 ± 0.2	165 ± 20	159 ± 20	38.1 ± 0.4	37.8 ± 0.4	33.1 ± 0.8	32.4 ± 0.5	37.1 ± 0.4	36.7 ± 0.5	6.4 ± 1.6	5.3 ± 1.5
HST2	0.81 ± 0.2*	156 ± 12*	150 ± 14*	37.8 ± 0.3	37.6 ± 0.3	33.7 ± 1.3	33.3 ± 1.1	37.0 ± 0.3	36.8 ± 0.4	5.6 ± 0.9*	4.8 ± 1.2

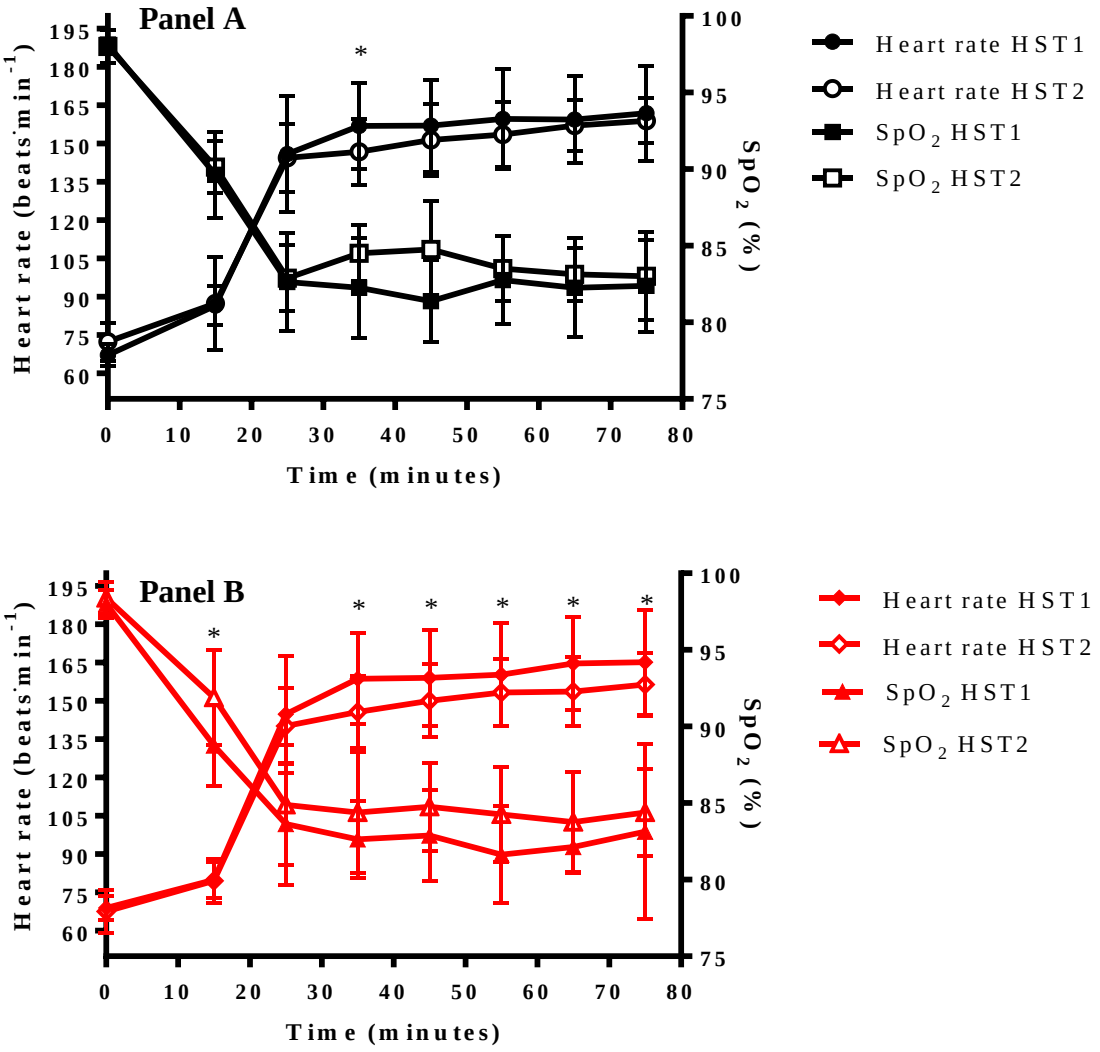

Figure 5.5. Mean ± SD heart rate and SpO_2 responses to HST 1 and HST in the CON group (*n* = 8, Panel A) and STHA group (*n* = 8, Panel B). * = differences between HST1 and HST2 ($P < 0.05$).

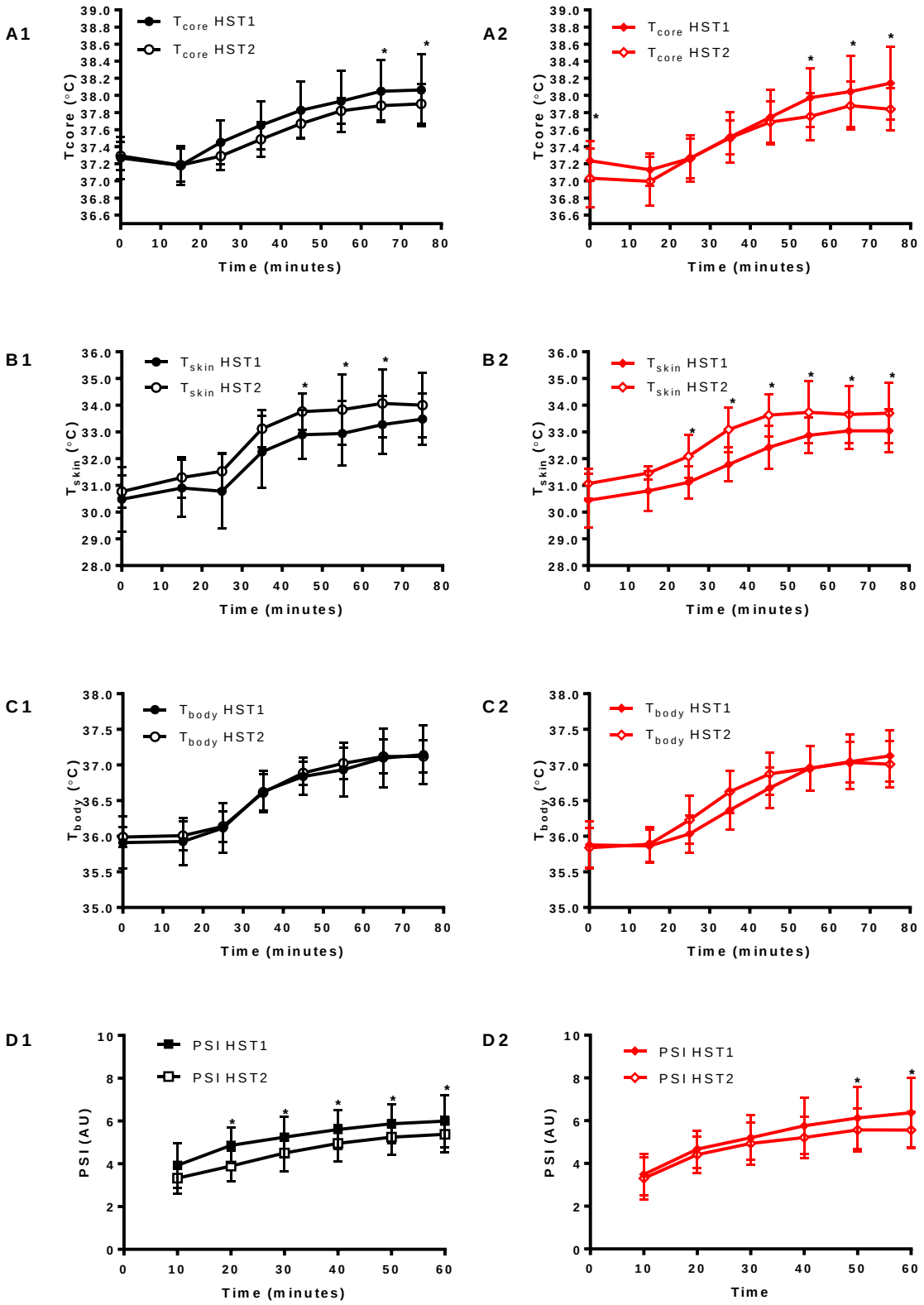

Figure 5.6. Mean ± SD T_{core}, Tskin,Tbody and PSI responses to HST1 and HST2 for CON (*n* = 8) and STHA (*n* = 8). * difference between HST1 and HST2.

5.3.6 Respiratory responses to Hypoxic stress test

There was no main effect of trial on minute ventilation (BTPS; F = 1.383, P = 0.259) oxygen consumption (F = 0.581, P = 0.459), carbon dioxide production (F = 0.391, P = 0.518), RER (F = 0.499, P = 0.492), or ventilatory equivalents (F = 0.441, P =0.497). Resting and end of exercise data are presented in Table 5.4.

5.3.6 Subjective responses to the HST

RPE was lower during HST2 compared to during HST1 (main effect for trial, F = 5.129, P = 0.04) although no trial x group interaction was observed (F = 2.111, P = 0.168; Table 5.4). Thermal sensation was lower during HST2 compared to HST1 (main effect for trial, F = 6.601, P = 0.022) with no trial x group or trial x group x time interaction (P > 0.05; Table 5.4).

5.3.7 mHSP72 responses to hypoxic stress test

The initial HST produced a significant increase in mHSP72 in CON (134 ± 51%) and STHA (139 ± 37%). This response was not significantly different between groups (P > 0.05). STHA resulted in an increase in resting mHSP72 prior HST2 in the (128 ± 26%, P < 0.05). Resting mHSP72 was unchanged in CON (103 ± 27%). The mHSP72 response to HST2 was similar to HST1 in CON (HST1 137 ± 20%; HST2 148 ± 30%). STHA had an attenuated response following HST2 compared to HST1 (HST1 139 ± 37%; HST2 98 ± 12%; P < 0.05). mHSP72 was significantly lower post exercise in the STHA group compared to the CON group (P < 0.05) (Figure 5.7)**.**

Table 5.4. Respiratory and subjective responses to the HST. Data are means ± SD. * = difference between HST1 and HST2 ($P < 0.05$).

	CON		STHA	
	HST 1	HST 2	HST 1	HST 2
Cardio-respiratory				
$\dot{V}_E$ (L•min^{-1} BTPS)	63.4 ±12.2	66.2 ± 16.3	70.3 ±19.0	65 ± 13.5
$\dot{V}O_2$ (L•min^{-1} STPD)	1.95 ± 0.32	2.07 ± 0.21	2.06 ± 0.31	2.04 ± 0.40
$\dot{V}CO_2$ (L•min^{-1} STPD)	1.83 ± 0.42	1.92 ± 0.34	1.94 ± 0.42	1.85 ± 0.3
RER	0.94 ± 0.10	0.93 ± 0.02	0.94 ± 0.09	0.91 ± 0.07
$\dot{V}_E / \dot{V}O_2$ (L•min^{-1} STPD)	33.0 ± 5.0	31.8 ± 3.4	33.8 ± 5.5	31.9 ± 2.8
$\dot{V}_E / \dot{V}CO_2$ (L•min^{-1} STPD)	35.1 ± 2.5	34.3 ± 3.6	35.9 ± 4.6	36.1 ± 5.5
Subjective				
RPE	16.0 ± 2.6	15.3 ± 2.5*	16.1 ± 2.6	13.8 ± 1.9*
TS	5.9 ± 1.0	5.6 ± 0.7*	6.1 ± 0.8	5.3 ± 1.3*

Significant correlations were observed for the pre-exercise mHSP72 expression and the percentage change in expression following exercise for HST1 in both CON and STHA (Figure 5.8, Panel A), and were also present during HST2 for CON (Panel B). This relationship was weakened post HST2 following STHA (Figure 5.8 panel B).

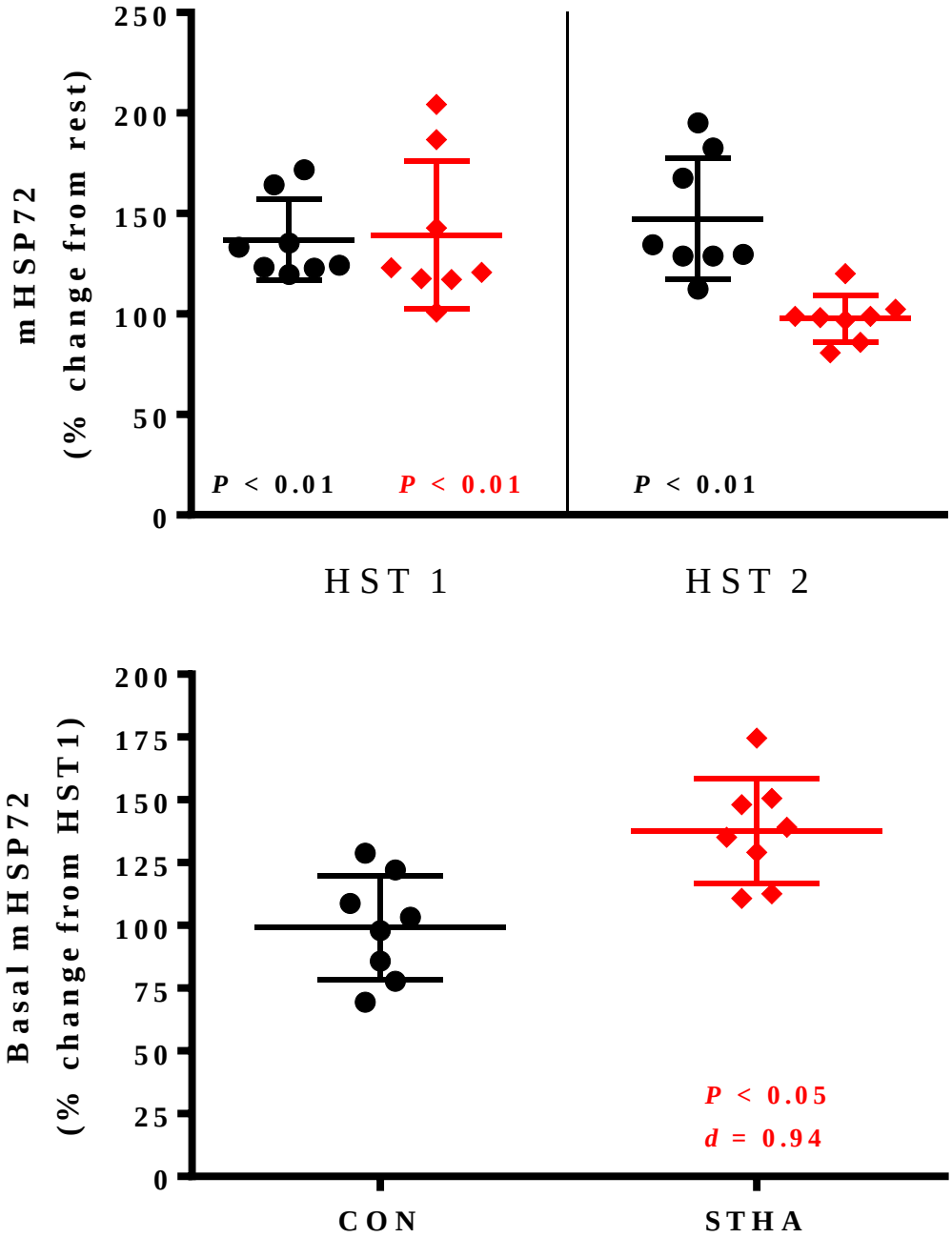

Figure 5.7. Fold change in mHSP72 immediately after HST1 and HST2 (Panel A) for CON (black dots, n = 8) and STHA (red dots, n = 8). Basal mHSP72 was increased prior to the onset of HST in the STHA group (Panel B, $P < 0.05$, $d = 0.94$)

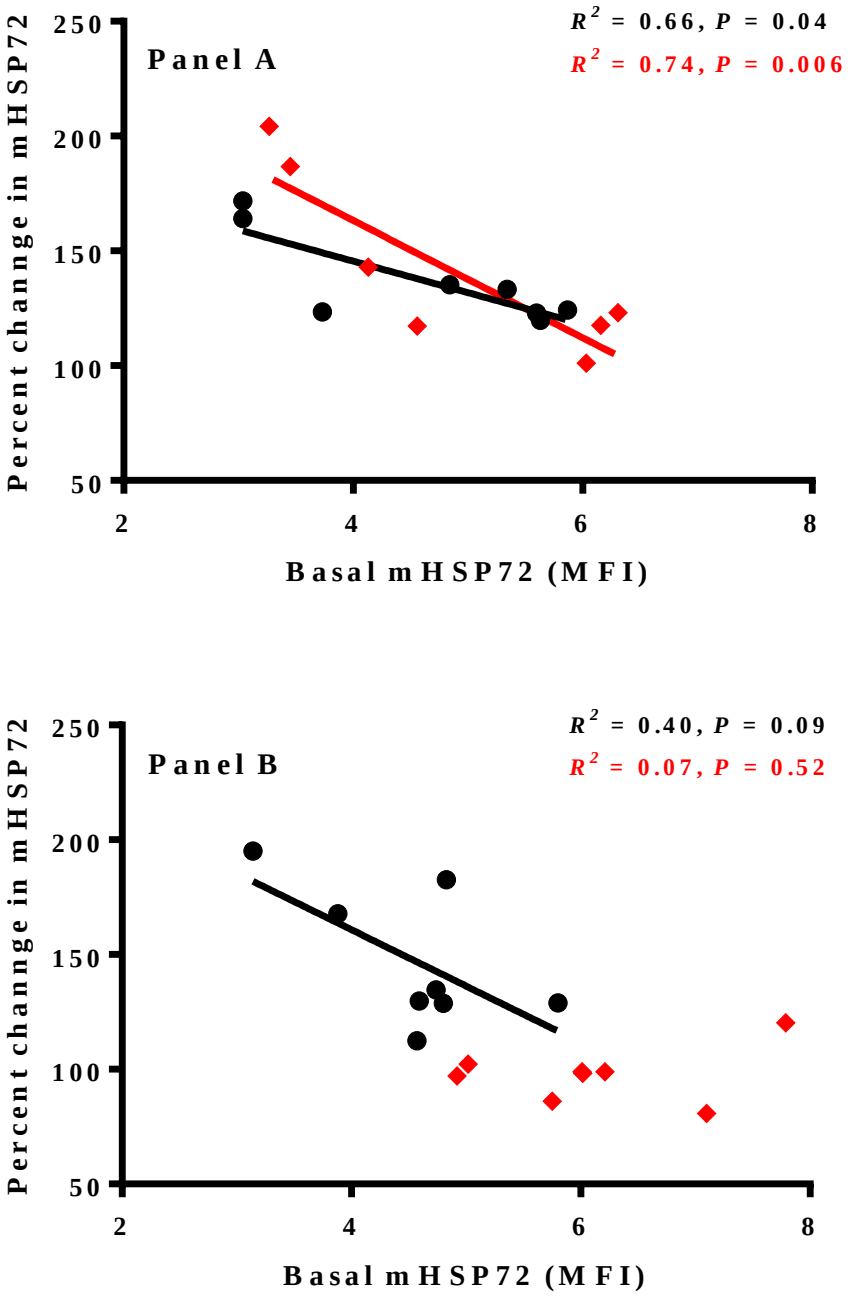

Figure 5.8. Relationship between pre-exercise expression of mHSP72 and mHSP72 inducibility (percent change from pre exercise values) following the HST. Linear regression showed a significant negative relationship between variables for CON (Panel A, black dots, R = -0.82, R^2 = 0.66, P = 0.04) and STHA (Panel A, red dots, R = -0.86, R^2 = 0.74, P = 0.006) in HST1. This relationship was still present in HST2 for CON (Panel B, black dots**,** R = -0.63, R^2 = 0.40, P = 0.09), but not observable post HST2 for STHA (Panel B, red dots**,** R = 0.27, R^2 = 0.07, P = 0.52).

5.3.8 Circulating HSP72 responses to the hypoxic stress test.

One participant in the control group was below the detection limit of the assay at rest throughout all trials and was removed from the analysis. eHSP72 increased to a similar magnitude following HST1 and HST2 in the CON group (HST1: 0.35 ± 0.29ng•ml.$^{-1}$; HST2: 0.55 ± 0.40ng•ml^{-1}, Figure 5.9). The acclimation period had no effect on the eHSP72 response to hypoxia (main effect for trial F = 0.87 P = 0.37) in the STHA group (HST1: 0.51 ± 0.35ng•ml^{-1}; HST2 0.32 ± 0.34ng•ml^{-1}, Figure 5.9). The eHSP72 response to the HST1 was smaller and less variable compared to the response to an acute heat stressor (HA1: 1.06 ± 0.74ng•ml^{-1}; HST1 0.51 ± 0.35ng•ml^{-1}).

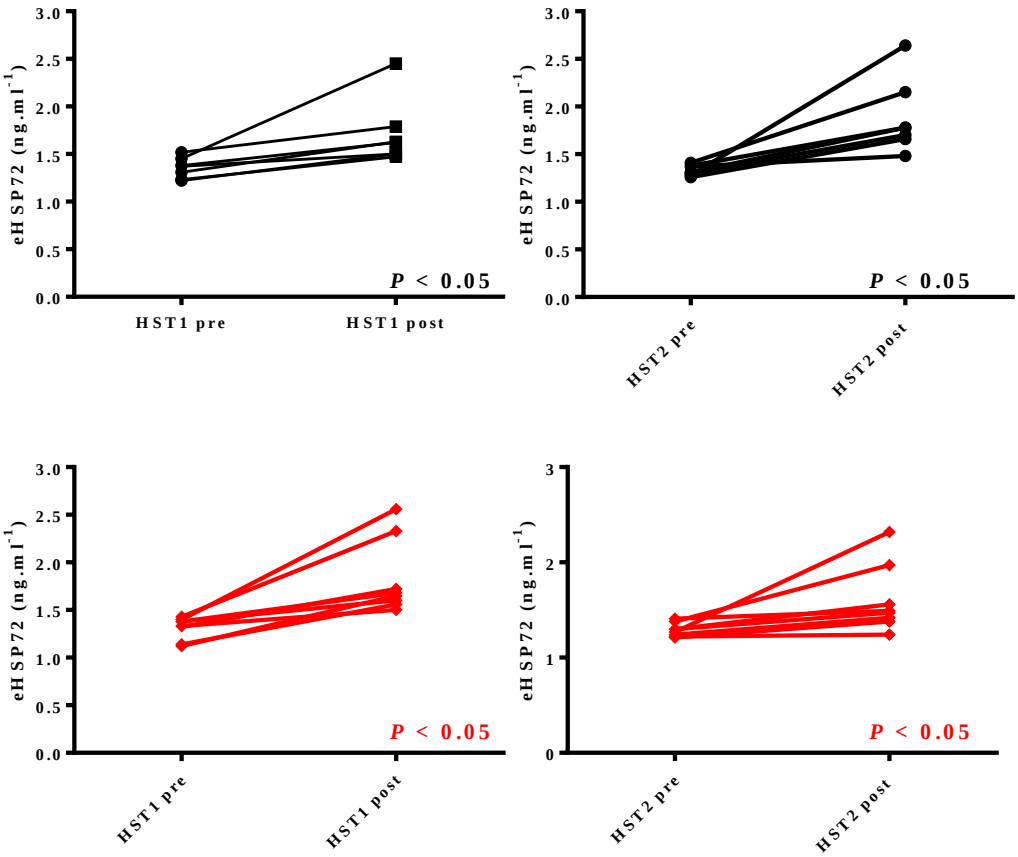

Figure 5.9. eHSP72 responses before and immediately after hypoxic stress test 1 (HST1) and hypoxic stress test 2 (HST2) for CON (*n* = 7, Panel A, black lines) and STHA (*n* = 8, Panel B, red lines). Lines represent the individual participant responses.

5.4 Discussion

The aim of this study was to determine whether the initial 3 days of heat acclimation would improve physiological and cellular tolerance to a subsequent acute exposure to normobaric hypoxia. To the author's knowledge this is the first *in vivo* human study examining the phenomenon of cross-acclimation between heat and hypoxic stressors during the initial phase of heat acclimation. This study has shown that 3 repeated one hour exposures to acute heat stress were associated with improvements to cellular tolerance alongside favourable reductions in physiological strain during an acute exposure to moderate hypoxia.

5.4.1 Attainment of heat acclimation

The STHA group demonstrated the classical improvements in cardiac stability, reductions in exercising core temperature, reduced physiological strain and increased sweat rates, alongside increases in basal mHSP72 (Table 5.2; Marshal et al., 2006; Garrett et al., 2008). In contrast 3 repeated bouts of aerobic exercise in a thermoneutral and normoxic environment did not induce any significant alterations in cardiovascular, thermal, mHSP72 or eHSP72 variables in the CON group (Table 5.2; Figures 5.2 and 5.4).

5.4.2 Monocyte and circulating HSP72 responses to heat acclimation

Prior to the commencement of the 3-day acclimation period (7 days after HST1), basal mHSP72 values had returned to those observed pre HST1 in both groups (Figure 5.2). This is experimentally important, as the magnitude of HSP72 response to a stressful insult has been shown to be proportional to its basal content prior to stressful insults (Vince et al. 2010). Both mHSP72 and eHSP72 were significantly increased from baseline following the initial day of heat exposure in the STHA group as previously observed following acute heat exposure (Figures 5.2 and 5.4; Fehrenbach et al., 2005). The mHSP72 response occurred to a similar

magnitude and over a similar time course to those reported in other studies (Fehrenbach et al. 2005, Fehrenbach et al. 2001). After 2 acclimation days mHSP72 remained elevated (30 ± 23%) prior to the onset of HA3 as previously observed at similar time points of a heat acclimation period (Marshall et al., 2007; Hom et al., 2012). A 40% increase in basal lymphocyte HSP72 has been observed after 4 days of walking for 90 minutes in 33°C, 30-50% humidity (Hom et al., 2012), and increases of ~ 30% in mHSP72 observed 24 hours after 60 running in hot conditions (60 mins at 90% of lactate threshold velocity, 28°C). A negative relationship between basal mHSP72 and the magnitude in post exercise change was observed in the current study (Figure 5.3, panel B) on the first day of the heat acclimation period. This is in line with the accepted inverse relationship between basal mHSP72 and its induction via a stressor (Vince et al., 2010). Following 3 days of exercising heat exposure this relationship was weakened (Figure 5.3, panel B). The increase in basal levels during this present investigation blunted the mHSP72 response (94 ± 14 %) which is an accepted characteristic in the shift towards a heat acclimated state (Magalhães et al. 2010, McClung et al. 2008, Yamada et al. 2007) and indicative of increased cellular tolerance (Horowitz et al., 2007; Madden et al., 2008).

Resting eHSP72 has previously been shown to decrease following the initial two days (Marshall, Ferguson and Nimmo 2006), 5 days (Kresfelder, Claassen and Cronje 2006) and 11 days (Magalhães et al. 2010) of exercise-heat acclimation. Data in the current investigation appear to follow this trend (Figure 5.3), but did not reach statistical significance. Two participants in the heat group recorded levels below the detection level of the assay at rest on day 3 of the acclimation period. It is worth noting that these participants displayed the largest post exercise changes in eHSP72 immediately after HA1. Post exercise eHSP72 was still significantly increased from rest on day 3, though the magnitude of this increase was

smaller. The inhibition of eHS72 release/production following exercise heat stress has previously been observed following a 5 day HA protocol, but only in participants that displayed classical signs of heat acclimation (reduced exercise HR and T_{rec} (Kresfelder, Claassen and Cronje 2006). A similar response was observed after a heat stress test (90mins running at 50% $\dot{V}O_{2\ peak}$ using a controlled hyperthermia protocol) after 11 days of heat acclimation (Magalhães et al. 2010). The release of eHSP72 has been shown to be both intensity and duration dependent (Périard et al. 2012), and also requires a minimum level of external stress to increase its appearance/release (Gibson et al. 2013). The 3 STHA period in this present study reduced the level of both thermal and cardiovascular strain, as evidenced by the reduced exercising HR and T_{core} (Table 5.2), thus it is possible that the external conditions experienced by participants on day 3 of acclimation were no longer sufficient to activate a similar response in eHSP72. That the CON group displayed minimal changes to these variables indicates that it is reasonable to conclude that the heat load experienced by the STHA group was of sufficient magnitude to induce a shift towards the acclimated phenotype.

5.4.3 Physiological responses to the HST

It is relatively common for both athletes and military personal to be exposed to moderate altitude, and to be expected to perform physical tasks without undergoing prior acclimatization. It is well established that even in the moderate altitude conditions studied herein, exercise performance, psychomotor performance and cognitive function is reduced (Fulco, Rock and Cymerman 1998, Kourtidou-Papadeli et al. 2008, Wagner et al. 2011). Adaptation to altitude requires approximately 14 days of residence at high altitude, with molecular adaptations serving both to improve oxygen delivery to cells and to maintain the structure and function of cells and organs (Horowitz 2007). However, in scenarios where rapid deployment of troops is necessary a 14 day time frame is logistically problematic.

Therefore, from a practical perspective, shorter interventions that can maintain or improve performance at altitude are of interest. Heat acclimation reduces oxygen uptake, induces glycogen sparing, increases plasma volume and improves myocardial efficiency and contraction, thereby reducing the stress on the cardiovascular system (Epstein et al. 2010, Horowitz, Parnes and Hasin 1993, Pandolf 1998). Despite the hematological and respiratory mechanisms of adaptation differing between heat and hypoxia, the increased physiological efficacy that is seen following a period of heat acclimation (Lorenzo et al. 2010) and shared molecular adaptations to the heat shock protein network, possibly mediated by the hypoxia-inducible factor (HIF-1α) (Maloyan, Palmon and Horowitz 1999, Maloyan et al. 2005), may point to a cross-acclimation effect being attainable. However, experiments that have explored the cellular and molecular responses to preconditioning/cross acclimation interventions have done so without due consideration of the whole body physiological implications arising from any observed adaptation, or increased cellular tolerance (e.g. Taylor et al., 2012). The present study attempted to determine if any heat acclimation-induced alterations in the cellular stress response elicited measureable improvements in physiological tolerance when exposed to a subsequent, acute exercising hypoxic challenge. The findings of this present investigation point to the possibility that a prior period of exercise-heat stress may be associated with beneficial physiological outcomes when later exposed to a period of acute hypoxic work. The reduced exercising HR in the STHA group, combined with an elevated SpO_2 would indicate that this group was more tolerant to acute hypoxia after the acclimation period. These preliminary results indicate that further work examining heat acclimation and hypoxic performance is warranted. The reductions in mean and peak exercising HR of approximately 9 beats•min^{-1} may indicate an increased capacity for work in these conditions, however follow up studies involving a hypoxic-adaptation group would allow the efficacy of time matched acclimation protocols to be assessed.

5.4.4 Monocyte HSP72 responses to the hypoxic stress test

The potential for two stressors that share several molecular and metabolic responses to invoke cross-acclimation at the cellular and whole body level has also been well examined in animals *in vivo* (Assayag et al. 2010, Horowitz et al. 2004, Kodesh and Horowitz 2010). In accordance with previous research (Taylor et al. 2010, Taylor et al. 2010, Taylor et al. 2012), mHSP72 increased following the first acute hypoxia exposure in both the CON (133 ± 37%) and STHA (139 ± 17%) groups, and to a similar magnitude previously reported in participants with similar physical characteristics (Chapter 4). It is likely that the increased oxidative stress associated with acute hypoxia, and the subsequent damage to membrane structures and proteins act as stimuli for HSP72 induction during hypoxia (Kulkarni, Kuppusamy and Parinandi 2007, Semenza 2000, Taylor et al. 2010). In the present study the post exercise values are lower than those reported by an acute-resting intervention (Taylor et al., 2010). This difference is likely due to the different participant characteristics and large inter-individual variation in the mHSP72 response to stressors.

The heat stress induced increase in mHSP72, in the current study, persisted for 48 hours after the final HA session in the STHA group and was elevated prior to HST2 (128 ± 26%; Figure 5.6). This prolonged elevation in mHSP72 after removal from repeated daily stress exposures has previously been observed 48 hours after 10 consecutive daily, 75-minute, passive exposures to hypoxia in healthy humans (Taylor et al., 2012). Taylor et al. (2012) observed increases in mHSP72 of ≈ 30% per day from baseline for the first 5 days of daily repeated hypoxic exposure. This decreased to a 16% increase per day for the final 5 days, representing a total ≈ 200% increase in mHSP72 over the 10-day period. Resting mHSP72 remained elevated 48 hours after the final exposure (≈225%). However Taylor et al. (2012) found that during the initial 3 days of the hypoxic acclimation period, mHSP72 was increased by ≈ 50%

from baseline. This magnitude in mHSP72 induction following the early stages of repeated hypoxic exposures is not dissimilar to that seen in the current experiment as a response to repeated exercising heat exposure (≈ 30% increase in baseline on day 3). It is worth noting that participants in this present investigation began to show a blunting in the mHSP72 on the third day of acclimation, whereas Taylor et al., (2012) demonstrated continual, modest increases in mHSP72 following passive hypoxic exposures on days, 4, 5, and 10. This indicates that the internal strain placed on participants in the present investigation may have reached an earlier ceiling for the level of strain required to produce further mHSP72. Increased basal HSP72 is a well-defined characteristic of both acquired thermotolerance (Kuennen et al., 2010; Hom et al., 2012), and improved cellular tolerance to repeated hypoxic exposures (Taylor et al, 2010) in humans. Thus it is not surprising that a stressor that invokes the HSR and leads to the subsequent increase in basal mHSP72 would lead to improved cellular tolerance to a second, novel stressor, in this instance acute hypoxia. A relationship between pre-exercise mHSP72 and the magnitude of mHSP72 change was seen in both groups following HST1, and in the control group following HST2 (Figure 5.7). However the acclimation period appeared to affect this relationship in the heat group, further indicating heat acclimation induced alterations in the expression kinetics of intracellular HSP72.

The stress-induced increases in mHSP72 after HST2 were attenuated in the STHA group compared to the post HST1 values. The mechanisms by which an increase in basal levels of mHSP72 may inhibit its own expression is related to HSP72 binding to heat shock transcription factor 1 (HSF1) (Morimoto, 1998). In unstressed cells HSF1 is bound to HSP72. Under stressful conditions HSP72 binds to denatured proteins, freeing HSF1. HSF1 trimerises and relocates to the nucleus where it binds to the heat shock element (HSE), initiation transcription of HSP72. When sufficient HSP72 has been produced to deal with the

rigors of the stressor, HSP72 rebinds to the HSF and halts further transcription (Morimoto, 1998). It is possible that the 3-day HA period induced increases in mHSP72 elevated the cellular stress required to induce further HSF1 activation. The induction of mHSP72 via a short term HA period was sufficient to allow the cells to cope with the hypoxic challenge, maintaining normal cell function and homeostasis. Increased bio-available HSP72 is implicitly linked to increased cellular protection (Garramone et al., 1994; Lepore et al., 2000; McArdle et al., 2004). Heat shocked cells displaying increased HSP72exporession demonstrate HSP72 dependant tolerance to the necrotic effects of hypoxia in comparison to non-heat shocked cells (Kiang et al., 1996) Whilst it is not proposed that whole body preconditioning and cellular tolerance has been conferred from the initial phase of acclimation studied, these results do warrant further investigation. Without parallel measures in skeletal muscle, the whole body responses during the initial phase of acclimation cannot be fully explored, and thus this response requires further investigation. The inclusion of a normothermic-exercise control group allowed for the effects of exercise and heat to be separated, which has been a design problem with other studies investigating the heat shock response in humans. Exercise in the absence of an external heat stress led to small, non-significant (10%) increases in mHSP72, similar to those previously reported for similar work bouts (Peart et al. 2011). The inclusion of a hypoxic group in future studies would also allow differences in expression kinetics to be quantified by the two divergent physiological stressors, and a further exploration of heat-mediated tolerance to hypoxia.

5.4.5 Circulating HSP72 responses to the HST

Plasma HSP72 also increased following the HST in both sets of participants. The current study is the first to measure eHSP72 in response to an acute exercising hypoxic exposure in humans. Post HST1, eHSP72 increased significantly in the STHA group (Figure 5.7),

whereas normoxic exercise failed to induce any change in this variable in the control group. It is likely that the normoxic exercise challenge failed to invoke a significant endogenous external stressor to stimulate the release of eHSP72 in this group. In contrast, the level of thermal strain experienced by the heat group presented a greater physiological stress than experienced during the level of acute hypoxia studied in this investigation. This is perhaps not surprising, as the rate of core temperature increase and delta change in core temperature have been found to be important external moderators in altering eHSP72 expression (Periard et al., 2012; Gibson et al., 2013). However, while these are important factors invoking a change in circulating levels of this protein, other factors have also been shown to be important. For example, both intensity and duration of exercise affect eHSP72 concentrations when work is performed in thermoneutral conditions (Ferenbach al., 2005), with the addition of a thermal stressor increasing this response (Marshall et al., 2006). Therefore, the increase in eHSP72 following HST1 may reflect the increase in relative work intensity. The absolute level of work used (50%normoxic $\dot{V}O_{2\ peak}$) has been shown in our laboratory, using participants of similar physiological characteristics and training background, to correspond to 78% of hypoxic $\dot{V}O_{2\ peak}$ (Lee et al, 2013). In conditions of matched heat stress (40C, 50%RH) but differing work loads (60 and 75% $\dot{V}O_{2\ peak}$), no difference in post exercise eHSP72 was observed, despite markedly different times to exhaustion (60%: 58.9 ± 10.9mins; 75%: 27.2 ± 9.0 mins) (Periard et al., 2012). The magnitude of eHSP72 increase following the HST was lower than observed by Periard et al., (2012), who reported increases in eHSP72 of approximately 2ng•ml^{-1}. In order to determine the specific effects of hypoxia *per se* on eHSP72, absolute and relative levels of work would need to be matched in both normoxic and hypoxic conditions. Such a matching of work levels was beyond the scope of this present investigation, thus the response of eHSP72 to acute bouts of moderate hypoxia warrants further investigation.

Experimental limitations are seen from the small number of participants in each experimental group. During the acclimation period there was considerable variation in the thermoregulatory response between participants. Future studies in which thermal strain is manipulated using the passive isothermic hyperthermia/thermal clamp technique to elevate and maintain T_{core} above 38.5° C (Gibson et al., 2013) throughout each acclimation session may induce a greater level of adaptation, and possibly reduce the large inter-individual variation in the eHSP72 response. A group undergoing hypoxic acclimation would have allowed the acclimatory responses to hypoxia to be compared to heat acclimation. This would have provided useful information regarding the efficacy of the heat acclimation period in inducing hypoxic tolerance, although matching for work intensity between heat and hypoxia would prove experimentally difficult.

5.5. Conclusion

In conclusion, 3 consecutive exercise-heat exposures at a fixed work rate resulted in increased levels of monocyte HSP72 in humans and affected the expression characteristics of this protein in subsequent exposures to acute heat stress and acute hypoxia. This improved capacity of the chaperone system attenuated the cellular stress response to hypoxia, although this resulted in only small changes in cardiovascular and thermoregulatory responses to subsequent hypoxia.

These data (Chapter 4, Chapter 5) indicate that acute exercising heat exposures of up to 90 minutes below the anaerobic threshold can induce a preconditioning effect in humans during submaximal hypoxic exercise 24 hours later (Chapter 4). The present chapter indicates that a 3-day fixed work-rate acclimation regimen for up to 60 minutes per day induces the initial phase of heat acclimation (STHA) as indicated by reduced exercise HR and T_{core}. These adaptations induced a similar preconditioning/cross acclimatory effect upon an exercising

hypoxic exposure 48 hours after withdrawal from the acclimation stressor. As the fixed work acclimation protocol is an easy to implement intervention it would be pertinent to determine whether a longer, more traditional period of heat acclimation (10 days, Castle et al., 2011) would induce a greater magnitude of cross-acclimation upon a later acute hypoxic exposure. The effects of a prior acclimation period on physical performance also warrant further investigation.

Chapter 6. The effects of 10 daily 1-hour heat or normobaric hypoxic cycle exercise on subsequent exercise tolerance and cycling performance in normobaric hypoxia.

This experimental chapter has formed the basis of the publication detailed below:

B. J Lee, V. Cox, A. Hussain, R. E Owen, A. Miller, T. Peplar, R. S James & C.D. Thake (*In preparation*). The effects of 10 daily 1-hour heat or normobaric hypoxic cycle exercise on subsequent exercise tolerance and cycling performance in normobaric hypoxia. *Experimental Physiology.*

6.1 Introduction

It is common for military recruits, athletes and those pursuing recreational activities to be exposed to high altitudes without a prior period of acclimatization (Fulco et al., 1998). Unacclimatized soldiers have been shown to experience decrements in physiological performance at elevations above 1500m above sea level (Fulco et al., 1998). During combat operations in Afghanistan, high altitude operations caused "combat ineffectiveness" led to "aborted missions from altitude sickness" and situations whereby soldiers could not pursue the enemy (Rodway and Muza, 2011). Unique to military operations is the requirement for rapid deployment of personnel to high altitude environments. Thus the traditional staging of mountaineering expeditions and slow ascent to allow for acclimatization is not feasible due to the long length of time required to achieve an acclimatory state. One method that has been utilized by military and athletes alike to overcome this lengthy acclimation process is intermittent hypoxic exposures (IHE, Section 2.3.2.3). Whilst normobaric IHE has been shown to be effective in improving later performances in normobaric hypoxia (Biedleman et al., 2007; Muza et al., 2007) it appears to offer little to no benefit under conditions of "true" hypoxia (hypobaric hypoxia; Fulco et al., 2011). Thus alternative solutions to this issue are required.

Adaption to one environmental stressor can induce protective responses to other stressors (Maloyan et al., 2005; Tetievsky et al., 2007). For a positive cross-acclimatory response, both stressors must possess shared protective signaling pathways, such as those activated by the heat shock response (Horowitz et al., 2007). The majority of cross-acclimation work has been performed in rodent models (Levi et al., 1991; Levi et al., 1993; Horowitz et al 1999). These studies have consistently demonstrated that heat acclimation improves tolerance to conditions associated with reduced oxygen availability (Hiestand et al., 1955; Maloyan et al., 1999; Shein et al., 2005). The process of heat acclimation involves the accumulation of heat shock proteins, which increase in a multitude of tissues, suggesting that pre-existing elevation of HSP72 may allow continued work under high temperatures without the requirement of further de-novo HSP72 synthesis (Maloyan et al., 1999; McClung et al., 2008). Additionally, HIF-1α, the master regulator of O_2 homeostasis (Semenza, 1998), was significantly increased in both heart and brain tissue following a 30 day resting heat acclimation protocol in rats (Maloyan et al., 2005). Increased HIF-1α lead to the increased transcription of target genes (e.g. VEGF), suggesting that its increased expression serves a functional purpose (Section 2.7.2). The process of acclimation may "prime" the animal with the necessary protective tools to maintain cellular homeostasis during later stress exposure, however the role of HIF-1α in human heat acclimation, and subsequent hypoxic tolerance has not been examined.

The ability of acute heat exposure to affect physiological tolerance to later normobaric hypoxic exercise was indicated in Chapter 4; with reductions in exercising heart rate (~5 beats•min^{-1}) and physiological strain observed 24 hours after an exercise heat stressor. Once daily heat stress (1 hour per day for 3 days) also demonstrated physiological tolerance upon exposure to exercise in hypoxia when compared to normothermic controls (Chapter 5). The evocation of the HSR by heat and hypoxia in Chapter 4, and the post heat acclimation

(Chapter 5) blunting of mHSP72 after a later bout of hypoxic exercise would also suggest that cross acclimation between heat and hypoxia occurs in humans. It is unlikely that such elevations in mHSP72 alone will augment either maximal or submaximal exercise performance in either normoxia or hypoxia in humans. However, the extended physiological and cellular challenges to homeostasis faced under hypoxic conditions may mean that increases in basal HSP72 could confer, or provide a marker of, cellular tolerance to the biochemical rigors of such exposures, as demonstrated *in-vitro* (Shima et al., 2008). Determining cross-tolerance responses in humans could have important uses in military and athlete populations when planning rapid redeployments of troops or in the build up to an endurance event.

Therefore the aims of this study were to 1) assess the effect of repeated once daily exercise in hot or hypoxic conditions on basal and post exercise monocyte and extracellular HSP72, 2) determine the effect 10 days of repeated heat or hypoxic acclimation has on plasma HIF-1α, 3) determine whether 10 days of acclimation to either heat or hypoxia improve physiological and cellular tolerance to later hypoxic exercise, 4) determine whether a prior period of heat acclimation can improve cycling time-trial performance in hypoxic conditions. This study tested the following hypotheses:

1) Heat acclimation would induce a greater magnitude in basal mHSP72 increase throughout the intervention period compared to a control group and hypoxic acclimation group.
2) A prior increase in mHSP72 as a result of the acclimation intervention would attenuate the mHSP72 response to an acute exercise bout in hypoxia relative to the magnitude of the basal increase.
3) Hypoxic acclimation would increase plasma HIF-1α concentrations.

4) Acclimation to heat and hypoxia would improve time trial performance in acute hypoxia conditions.

6.2 Methods

6.2.1 Participant characteristics

26 healthy male participants (age 22 ± 4 years; height 1.76 ± 0.07m; body mass 71.8 ± 7.9kg) volunteered to take part in the present investigation, which was granted ethical approval by the Coventry University Ethics Committee. Participants attended the laboratory on 17 separate occasions (Figure 6.1). The initial visit involved the informed consent process, preliminary tests for body composition, lactate threshold, and peak oxygen uptake, as described in Section 3.6.1.

6.2.2 Experimental protocol

Participants completed 5 experimental stages over a 6-7 week period as displayed in Figure 6.1.

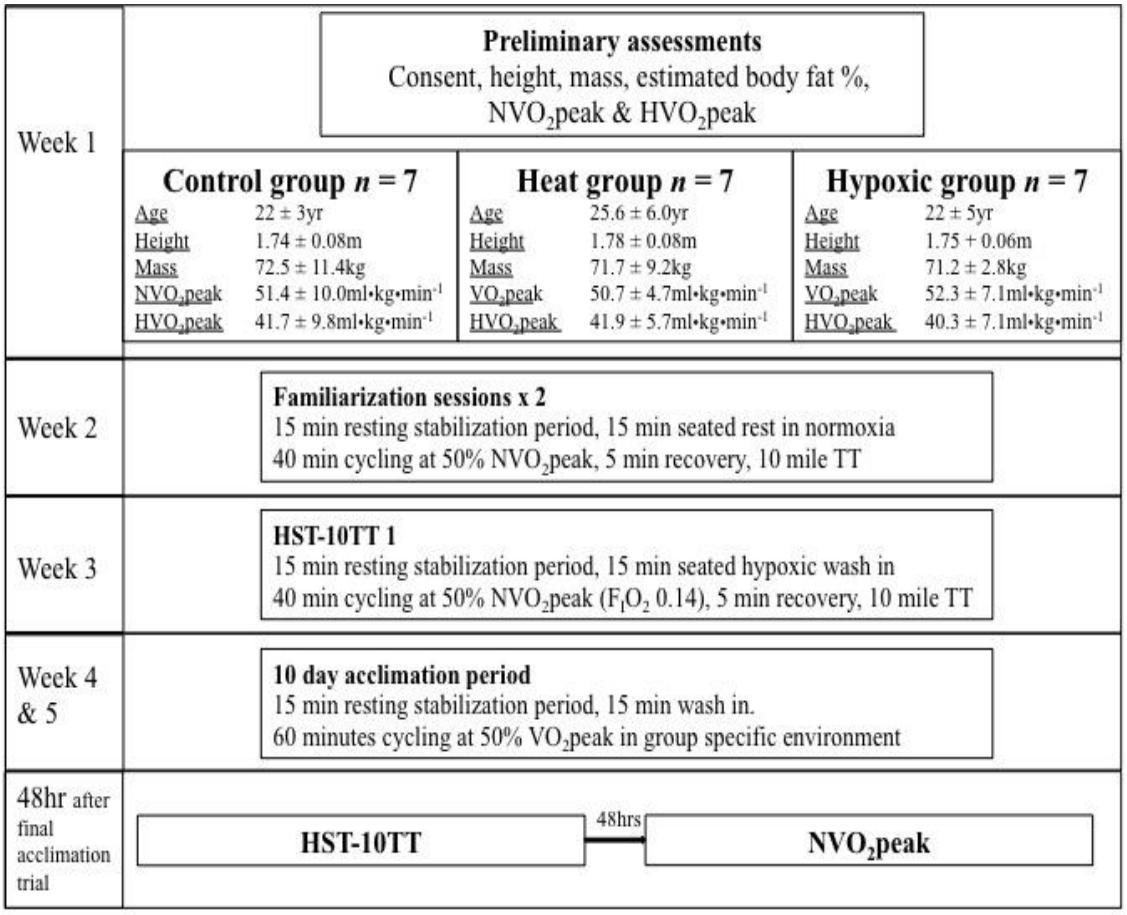

Figure 6.1. Experimental schematic. During the first week of testing participants completed a normoxic (N) and hypoxic (H) $\dot{V}O_2$peak test separated by at least 48 hours in a counterbalance manner. Following these tests participants were randomly assigned to 1 of 3 experimental groups. During week 2 participants completed two familiarisation sessions separated by at least 72 hours. At least 5 days after the final familiarisation session participants completed the pre-intervention HST and time trial prior to commencing the 10 day intervention period at least 7 days after the HST. 48 hours after the final intervention session participants returned to complete a final HST and $\dot{V}O_2$peak test.

6.2.2.1 Preliminary testing

Participants attended the laboratory on 2 separate occasions separated by a minimum of 48 hours. On the first preliminary session informed consent was obtained from participants. Each participant was asked to explain the trial design in their own words, and given the opportunity to ask any questions regarding any of the experimental sessions. If it was felt any participant had a poor understanding of the experimental process the study was explained to them further before consent was obtained.

Once consent was obtained the participants' height and weight were taken, and four skin-fold measurements were obtained by an International Society for the Advancement of Kinanthropometry (ISAK) qualified anthropometrist (Section 3.3). The SRM cycle ergometer was then set up for the participant, and handlebar and crank lengths, and saddle height and distance horizontally from the crank noted for all subsequent visits. The participant then began a lactate threshold and $\dot{V}O_{2\ peak}$ test in either normoxic conditions (F_IO_2 = 0.209), or hypoxic conditions (F_IO_2 = 0.14). This was performed in a counterbalanced fashion between preliminary visit 1 and preliminary visit 2. Details of this LT and $\dot{V}O_{2\ peak}$ protocol are in Section 3.6.1. Following the 2 preliminary visits, participants were matched to 1 of 3 groups, control (CON), long term heat acclimation (LTHA) or hypoxic acclimation (HA). The characteristics of each group are shown in Table 6.1.

Table 6.1. Mean ± SD participant characteristics. During preliminary testing 3 participants withdrew from the study, and a further 2 withdrew; 1 following the initial HST-10TT and 1 following 2 days of acclimation. Thus 3 groups of 7 participants completed the remainder of the study.

Group	Age (Years)	Height (m)	Weight (kg)	Body fat (%)	$N\dot{V}O_{2\ peak}$ ($mL \bullet kg^{-1} \bullet min^{-1}$)	$H\dot{V}O_{2\ peak}$ ($mL \bullet kg^{-1} \bullet min^{-1}$)
CON (*n* = 7)	22 ± 3	1.74 ± 0.08	72.5 ± 11.4	14.6 ± 4.3	51.4 ± 10.0	41.7 ± 9.8
HA (*n* = 7)	25 ± 6	1.78 ± 0.08	71.7 ± 9.2	14.3 ± 5.1	50.7 ± 4.7	41.9 ± 5.7
LTHA (*n* = 7)	22 ± 5	1.75 ± 0.06	71.2 ± 5.1	14.7 ± 2.8	52.3 ± 7.1	40.3 ± 7.1

6.2.2.2 Familiarisation visits

Two normoxic familiarisation visits were separated by a minimum of 72 hours. These sessions were performed to habituate participants to the time trial and minimize learning effects on performance (Section 3.11.5). Participants arrived between the hours of 0600 and 0800 for a 0800 or 1000 start, which was kept consistent for the remainder of the study. Participants were provided with a set breakfast (Complan meal replacement milkshake; 386 kcal, 15.6g protein, 44.4g carbohydrate; 16.4g fat, 400mL of water) and returned to the laboratory 2 hours (0800 or 1000 start) later to begin the experimental protocol. After voiding the bladder and providing a urine sample for the assessment of urine specific gravity (USG) and urine osmolality ($U_{OSMO),}$ participants weighed themselves nude, and inserted a rectal thermometer 10cm past the anal sphincter. Participants were seated and a heart rate monitor (Suunto T6c) fixed to the chest and skin temperature probes taped to the left thigh, calf, chest and triceps as described in Sections 3.4.1 and 3.7. At the end of a 15-minute stabilization period a 7mL venous blood sample was obtained from an antecubital vein into an EDTA treated vacutainer (Vacuette, Greiner Bio-One, UK, Section 3.12). The venous blood was

used to determine hemoglobin and hematocrit (Section 3.8.2), and a fingertip capillary sample was obtained to determine resting blood lactate (Section 3.8.1).

Participants were then seated on the SRM ergometer and a pneumatic fingercuff attached to the index finger of the right hand (Portapres Model-2, Finapres Medical Systems, Amsterdam) system, which was fixed via a sling at a height equivalent to the aorta via palpation of the third intercostal space. Participants then began the 15-minute wash-in period breathing room air through the gas-delivery system prior to commencing 40 minutes cycling at 50% normoxic $\dot{V}O_{2\ peak}$ (Section 3.11.2). HR, T_{core}, T_{skin}, $\dot{V}O_2$ $\dot{V}CO_2$, $\dot{V}_{E\ BTPS}$, RER, $\dot{Q}$, SV, RPE and TS were recorded every 10-minutes throughout the test. Following a 5-minute recovery period in which the Portapres and skin temperature probes were removed, participants completed a self-paced 16.1-km TT in the SRM open-ended test mode (Section 3.11.2). As the TT commenced an industrial fan ~1m away from the front of the ergometer was turned on, creating a windspeed of ~26km•hr^{-1} to simulate external conditions. This provided suboptimal convective cooling for TT completion time, though is more ecologically valid that cycling with no method of cooling. The only information available to the participant was distance covered. No verbal cues were provided to the participants by the researchers.

6.2.2.3 Hypoxic tolerance testing

At least 5 days after the second familiarization session participants returned to the laboratory at the same time of day to complete an identical testing procedure as described in Section 6.2.2.2 under hypoxic conditions (F_IO_2 = 0.14), the hypoxic tolerance test and 10 mile time trial (HST-10TT). A 7mL venous blood sample was obtained following the 15-minute normoxic rest period. Participants then began a 15-minute resting hypoxic wash-in period

whilst seated on the ergometer before completing the 40-minute HST. A further 7mL venous sample was collected immediately after the 40-minute exercise bout for the determination of mHSP72, eHSP72 and plasma HIF1-α (Section 3.12.2.1, 3.12.2.2 and 3.12.2.3 respectively) before the 10mile TT began.

6.2.2.4 Acclimation training

At least 7 days after the HST-10TT participants began the acclimation protocol. The CON group completed 10 days of cycling for 60 minutes at 50% normoxic $\dot{V}O_{2\ peak}$ in thermoneutral conditions (18.3 ± 2.0°C, 36.1 ± 4.3%RH), the LTHA group completed the same work in hot conditions (41.4 ± 1.4°C, 21.4 ± 3.4%RH) and the HA group completed the same work at an altitude equivalent of 3095m (F_IO_2 = 0.14, 18 ± 1.3°C, 35.3 ± 3.9%RH).

Prior to testing participants voided their bladder and provided a urine sample for the assessment of USG and U_{OSMO}, weighed themselves nude and then inserted the rectal thermometer as previously described (Section 3.11). Participants then returned to the laboratory and completed a 15-minute seated stabilization period in normoxic thermoneutral conditions. At the end of the stabilization period resting measure of T_{core}, HR and TS were collected and a fingertip capillary blood sample collected for assessment of blood lactate, Hb and Hct. Measures of HR, T_{core}, SpO_2, RPE, TS were taken, and PSI calculated every 10-minutes throughout exercise. At the end of exercise another fingertip capillary sample was collected, a final urine sample was collected for USG and U_{OSMO}, and nude body weight assessed in order to determine sweat rates. On acclimation days 1, 3, 5, 6, and 10 venous blood samples were collected for the determination of hemoglobin, hematocrit, and mHSP72. On blood sampling days participants arrived at the laboratory between the hours of 0600 and 0800 for a set breakfast as previously described. On days 2,4,7,8, and 9 testing was completed as near to each individuals initial starting time as possible (average of ± 3 hours).

6.2.2.5 Post acclimation testing

Participants returned to the laboratory 48 hours after the final acclimation session to complete a final HST-10TT. Between 24 and 48 hours after this test, participants returned to complete a normoxic lactate threshold and $\dot{V}O_{2\ peak}$ test to determine whether the 10 days of training had any affect on aerobic fitness parameters. As the time course of any cross-acclimation is not known, it was reasoned performing the final tests in this order would minimize the potential decay in acclimation.

6.2.3 Measures of cellular stress

Venous blood samples were collected as previously described (Section 3.12) before and after each HST for assessment of mHSP72, eHSP72 and plasma HIF-1α. During the acclimation period, samples were collected before and after exercise on days 1, 3, 5, 6 and 10 for assessment of mHSP72 via flow cytometry. The assay was completed 'live' and cells run within 2 hours of the sample collection.

6.2.4 Statistical analysis

Data are reported as means standard deviations for $n = 7$ in each experimental group, unless otherwise stated. All data were checked for normal distribution prior to analysis. Non-normally distributed variables were log-transformed to approximate normal distribution before applying the appropriate statistical test. Tests employing repeated measures were checked for sphericity before analysis with Mauchly's sphericity test. Where sphericity was broken, *P*-values were corrected using the Huynh-Feldt method. The alpha level was set at $P < 0.05$ for all tests.

6.2.4.1 Acclimation data

Data from the acclimation visits were analyzed using a one-way repeated measures ANOVA for comparison across days of acclimation, and significant differences identified using Tukey's post hoc HSD test. Days 1, 3, 5, 6, 10 were used in the analysis for each separate experimental group.

6.2.4.2 Hypoxic tolerance test and 10 mile TT

Physiological responses at rest and during exercise in HST1 and HST2 were analyzed using a 2 x 7 x 3 (trial x time x group) repeated measures ANOVA. Significant main effects were examined with Tukey HSD post hoc tests. Changes in resting plasma volume and pre – post exercise body mass between HST1 and HST2 were compared using paired *t*-tests.

6.2.4.3 Molecular responses to acclimation and HST

Monocyte HSP72, eHSP72 and HIF-1α responses to the acclimation period were examined using a 2 x 2 x 3 (trial x time x group) mixed repeated measures ANOVA. Significant main effects were examined with Tukey HSD post hoc tests. The mHSP72 responses throughout the acclimation period were analyzed using 5 x 2 x 3 (trial x time x group) mixed repeated measures ANOVA.

6.3 Results

6.3.1 Physiological and perceptual responses during the acclimation period

Participants in each group were hydrated prior to each session, as U_{OSMO} and USG was 553 ± 254mOsm•kg^{-1} and 1.004 ± 0.01, 465 ± 239mOms•kg^{-1} and 1.002 ± 0.01, 444 ± 234mOsm•kg^{-1} and 1.002 ± 0.01 for CON, LTHA and HA respectively. The 10-day

acclimation period had no effect on peak HR, T_{core}, PSI or PV in the CON group ($P > 0.05$). Following LTHA , peak HR during exercise was reduced from day 1 (165 ± 11beats•min^{-1}) to day 10 (145 ± 10 beats•min^{-1}; $F = 5.28$, $P = 0.03$). Peak T_{core} was reduced ($F = 5.245$, $P = 0.04$) from day 1 (38.73 ± 0.23°C) on day 6 (38.16 ± 0.31°C; $P < 0.05$) and day 10 (38.05 ± 0.23°C; $P < 0.05$) of acclimation. Concomitantly, peak PSI ($F = 5.309$, $P = 0.03$) was lower on day 6 ($P < 0.05$) and day 10 ($P < 0.01$) compared to day 1. Furthermore, participants in the LTHA group exhibited a PV expansion of 8.27 ± 3.52% by day 10 compared to day 1 ($F = 5.548$, $P = 0.003$). The majority of this expansion occurred early in the acclimation protocol, expanding by 2.85 ± 1.70% on day 3 ($P > 0.1$), 4.31 ± 2.29% on day 5 ($P < 0.1$), and 7.45 ± 3.51% on day 6 ($P < 0.05$). Thermal sensation was reduced from day 1 ($F = 7.533$, $P = 0.001$) on days 5, 6 and 10 ($P < 0.05$), but RPE was unaffected over the acclimation period ($F = 2.868$, $P = 0.107$).

The HA group had a reduced HR ($F = 6.802$, $P = 0.01$) on day 10 (141 ± 7 beats•min^{-1}) in comparison with day 1 (159 ± 17beats•min^{-1}; $P < 0.01$). Peak T_{core} ($F = 1.450$, $P = 0.248$) and PSI were not affected by the 10-day acclimation period ($F = 2.068$ $P = 0.117$). There was a trend for an increased SpO_2 ($F = 2.279$ $P = 0.09$) and a decreased PV ($F = 2.305$, $P = 0.08$). Pulmonary ventilation increased at rest and during exercise from day 1 to day 10 ($F = 3.51$, $P = 0.04$). Thermal sensation was unaffected ($F = 1.609$, $P = 0.205$), however RPE was reduced from day 1 ($F = 6.274$, $P = 0.009$) on days 5, 6 and 10 ($P < 0.05$). Individual data for peak physiological responses on day 1 and day 10 is shown in Table 6.2

6.3.1.2 Maximal aerobic capacity following the acclimation period

Table 6.3 displays each participant's normothermic, normoxic maximal aerobic capacity and lactate threshold before and after the 10-day intervention period. There were no differences in

peak power output, power at lactate threshold, and $\dot{V}O_{2\ peak}$ post acclimation for any of the experimental groups ($P > 0.05$).

Table 6.2. Mean ± SD peak physiological and perceptual responses during the 10-day acclimation period for CON (n = 7), LTHA (n = 7) and HA (n = 7). Pre acclimation plasma volume was 55.2 ± 2.3%, 55.0 ± 2.1% and 53.9 ± 2.1% for CON, LTHA and HA respectively.

	Peak HR (beats•min^{-1})	Peak T_{core} (°C)	Peak PSI	Peak RPE	Peak TS	ΔBM (kg)	ΔPV (%)
CON							
Day 1	137 ± 11	37.86 ± 0.25	4.0 ± 0.9	12.1 ± 0.7	5.0 ± 0.0	0.6 ± 0.2	-
Day 3	139 ± 15	37.99 ± 0.23	4.6 ± 0.8	11.6 ± 1.1	4.3 ± 0.8	0.6 ± 0.2	0.2 ± 4.7
Day 5	131 ± 17	37.85 ± 0.16	3.9 ± 1.1	11.1 ± 0.9	4.6 ± 0.5	0.9 ± 0.3	-1.0 ± 4.4
Day 6	136 ± 13	37.85 ± 0.09	4.5 ± 0.9	11.4 ± 1.3	4.4 ± 0.5	0.8 ± 0.5	3.3 ± 4.6
Day 10	137 ± 18	37.86 ± 0.23	4.2 ± 1.3	10.9 ± 0.9	4.6 ± 0.5	0.7 ± 0.3	2.4 ± 4.6
LTHA							
Day 1	165 ± 11	38.73 ± 0.23	7.4 ± 1.2	12.3 ± 2.2	6.7 ± 0.8	1.0 ± 0.6	-
Day 3	158 ± 17	38.22 ± 0.38	6.3 ± 1.2	12.9 ± 2.4	6.3 ± 0.5	1.2 ± 0.5	2.9 ± 1.7
Day 5	156 ± 13*	38.26 ± 0.37	6.2 ± 0.9	12.0 ± 2.2	5.9 ± 0.4*	1.1 ± 0.3	4.3 ± 2.3
Day 6	150 ± 9*	38.18 ± 0.29*	5.8 ± 0.9	11.9 ± 2.2	6.0 ± 0.0*	1.5 ± 0.3^	7.5 ± 3.6^
Day 10	145 ±10§	38.06 ± 0.23*	5.4 ± 0.5	11.3 ± 2.1	5.4 ± 0.5*	1.9 ± 0.3^	8.3 ± 3.5^
HA							
Day 1	159 ± 15	38.16 ± 0.46	5.9 ± 1.5	13.6 ± 2.9	5.0 ± 0.8	0.8 ± 0.3	-
Day 3	157 ± 10	38.14 ± 0.29	5.8 ± 1.1	12.7 ± 2.8	4.9 ± 0.7	0.7 ± 0.4	-2.9 ± 3.8
Day 5	158 ± 12	38.16 ± 0.38	6.1 ± 1.2	11.7 ± 2.4*	4.7 ± 0.8	0.7 ± 0.2	-3.1 ± 4.6
Day 6	154 ± 8	38.20 ± 0.25	5.9 ± 0.9	11.3 ± 2.3*	4.6 ± 0.5	0.7 ± 0.4	-3.6 ± 3.6
Day 10	142 ± 7§	37.92 ± 0.22	4.9 ± 0.5	10.6 ± 2.4§	4.3 ± 0.8	0.9 ± 0.3	-5.6 ± 3.7

* Significantly lower than day 1 ($P < 0.05$). § Significantly lower than day 1 ($P < 0.01$). ^ Significantly higher than day 1 ($P < 0.05$).

Table 6.3. Mean ± SD maximal aerobic capacity, peak power output, and power at lactate threshold pre and post acclimation for CON (*n* = 7) LTHA (*n* = 7) and HA (*n* = 7).

	$\dot{V}O_{2\ peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	Peak power (Watts)	Power at LT (Watts)
CON			
Pre acclimation	51.4 ± 10.0	282 ± 59	175 ± 44
Post acclimation	51.9 ± 8.6	285 ± 45	180 ± 51
LTHA			
Pre acclimation	52.3 ± 7.1	272 ± 34	179 ± 24
Post acclimation	53.4 ± 6.5	280 ± 39	182 ± 30
HA			
Pre acclimation	50.7 ± 4.7	281 ± 37	185 ± 27
Post acclimation	51.4 ± 5.4	287 ± 25	189 ± 38

6.3.1.2 mHSP72 responses to the acclimation period

The acclimation period induced no changes in mHSP72 at any time point for the CON group ($P > 0.05$). Basal mHSP72 was significantly increased throughout the acclimation period in both the LTHA and HA groups (trial x time x group interaction, $F = 2.549$, $P = 0.003$). Specifically, basal mHSP72 was increased on days 5, 6 ($P < 0.05$), and 10 ($P < 0.01$) compared to day 1 in the LTHA group, and on day 10 in the HA group ($P < 0.05$). Daily post exercise mHSP72 expression increased from resting expression on day 1 of LTHA ($P < 0.01$) and day 1 and 3 of HYA ($P < 0.05$; Figure 6.2).

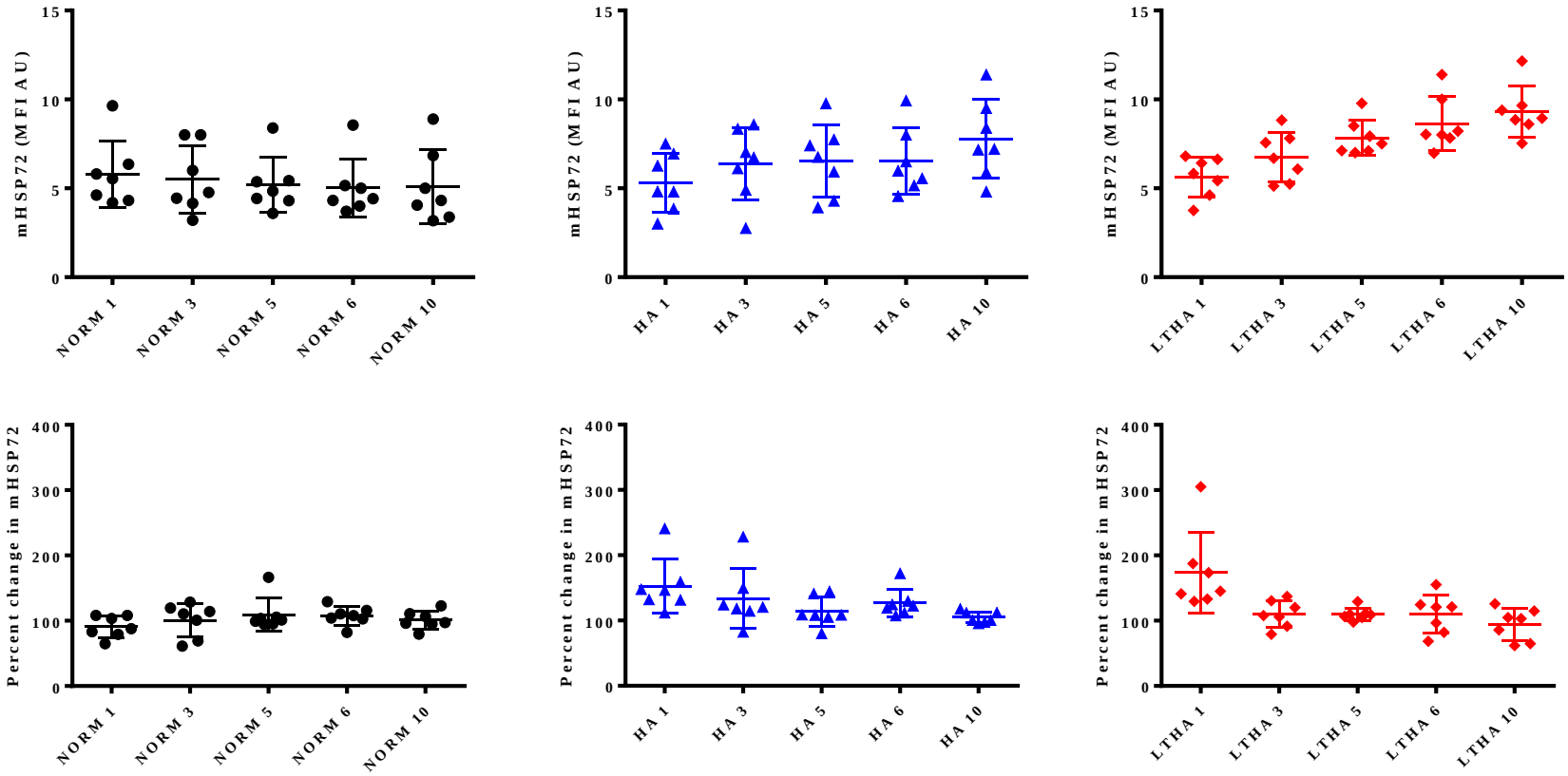

Figure 6.2. The top panels represent daily resting mHSP72 prior to the exercise bout. mHSP72 is expressed as MFI of the anti-HSP72 antibody in relation to the FITC matched negative control sample. Data is shown for NORM (black dots, n = 7) HA (blue dots, n = 7) and LTHA (red dots, n = 7). The bottom panels represent the fold change in mHSP72 post exercise in relation to the sessional baseline measurement of mHSP72. * significant difference from daily resting expression ($P < 0.05$). + significantly different from basal expression on day 1 of acclimation ($P < 0.05$).

6.3.2 Physiological and perceptual responses during the hypoxic stress test

Heart rate, SpO_2, T_{core}, mean T_{skin}, mean T_{body}, and PSI were not reduced following either CON group or HA ($P > 0.05$). Heart rate ($F = 5.95$ $P = 0.05$, Figure 6.3), T_{core} ($F = 8.99$, $P = 0.02$, Figure 6.4) and T_{body} (F = 6.60, $P = 0.04$, Figure 6.5) were reduced in HST2 following LTHA. Specifically, HR was reduced from 20 -40 minutes of exercise ($P < 0.05$), T_{core} was reduced at the end of the rest period ($P < 0.05$), and T_{core} and T_{body} reduced from 30 – 40 minutes of exercise ($P < 0.05$). PSI ($F = 3.64$, $P = 0.11$, Figure 6.6) and T_{skin} ($F = 3.42$, $P = 0.11$, Figure 6.7) showed a tendency to be reduced from HST1 ($F = 3.52$, $P = 0.09$). Plasma volume expansion was observed in the LTHA group prior to the onset of HST2 ($T = 8.356$, $P < 0.001$). There was no change in any of the measured mean or peak cardio-respiratory variables (Table 6.4). Pre to post body mass loss was increased in the LTHA group ($T = -5.095$, $P = 0.002$). A reduction in PV was evident in 6 out of 7 participants in the HA group ($T = 2.351$, $P = 0.057$). There were no changes in either the CON or HA group for PV and change in body mass ($P > 0.05$). Mean RPE was reduced during HST2 compared to HST1 in both the HA and LTHA group ($P < 0.05$). Thermal sensation was no different between HST1 and HST2 ($P > 0.05$).

6.3.3 mHSP72 expression before and after the HST

Prior to the acclimation intervention mHSP72 expression was increased immediately post exercise in CON (140 ± 14%), LTHA (158 ± 30%) and HA (145 ± 27%) (main effect for time ($F = 15.70$; $P < 0.01$). Following the acclimation period, mHSP72 expression remained unchanged in the CON group, and was elevated in the LTHA ($P < 0.01$) and HA group ($P < 0.05$) relative to the resting expression prior to completing

HST1. Post exercise expression of mHSP72 after HST2 was not different to post exercise expression following HST1 in any group ($P > 0.05$), but was only increased relative to HST resting expression in the CON group ($P < 0.05$). Figure 6.8 displays mHSP72 expression pre and post HST1 and HST2.

6.3.4 Extracellular HSP72

eHSP72 concentration is shown in Figure 6.9. eHSP72 was not different pre to post HST1 ($P > 0.05$) in any experimental group. Prior to the onset of HST2 eHSP72 had a tendency to be reduced in the LTHA group ($P = 0.09$), and was unchanged in CON and HA ($P > 0.05$).

6.3.5 Plasma HIF-1α

Plasma HIF-1α was increased following HST1 in all groups ($P < 0.05$; Figure 6.10), though considerable inter-individual variation was present, a post HST increase in HIF-1α was observed in all but 2 of the 21 participants. Basal HIF-1α remained unchanged from HST1 to HST2 in the CON group ($P > 0.05$), and was significantly increased in the HA group ($P < 0.01$) and LTHA group ($P < 0.05$). Accordingly, post HST2 failed to induce an increase in the HA group, with a small increase observed in the LTHA group ($P < 0.05$).

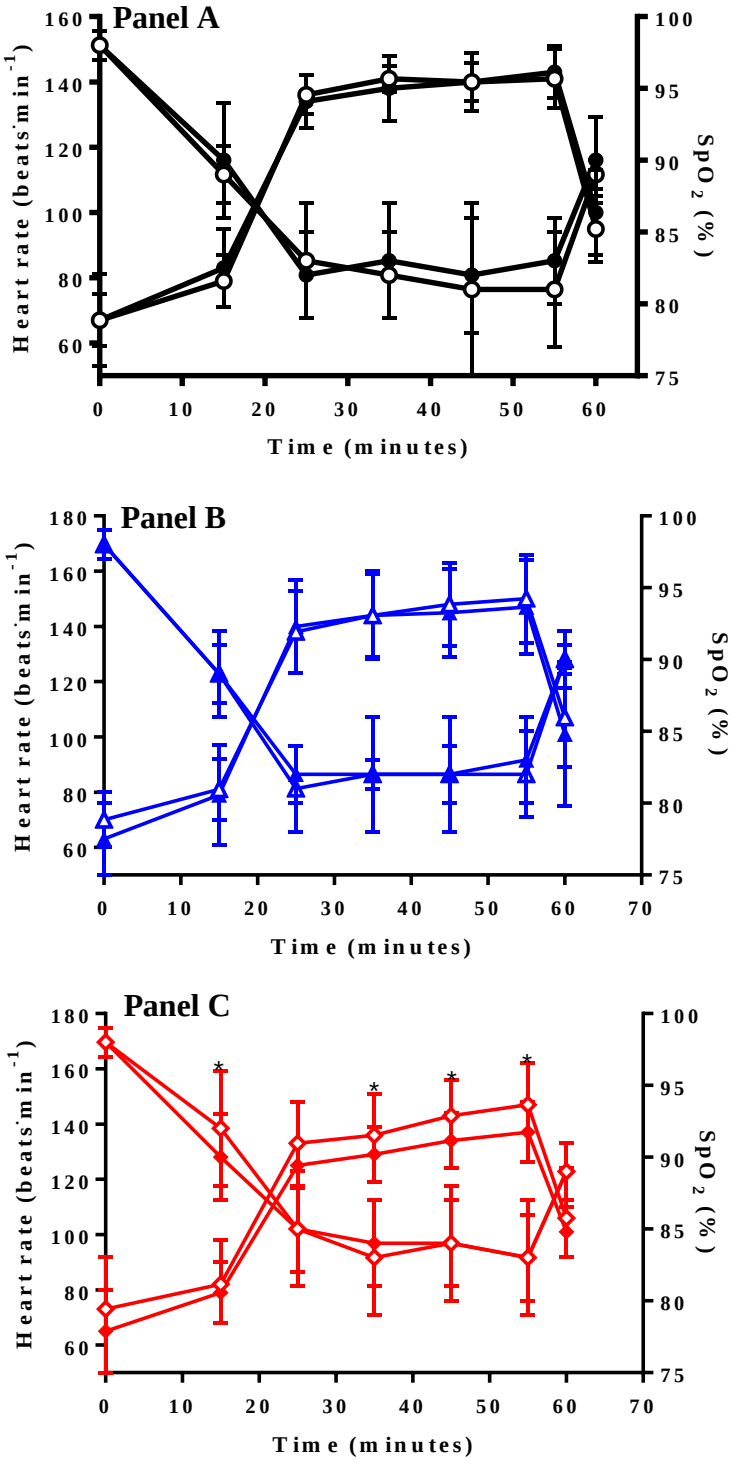

Figure 6.3 Heart rate (solid lines) and SpO_2 (dashed lines) during HST1 (clear markers) and HST2 (coloured markers) for CON (Panel A) LTHA (Panel B) and HA groups (Panel C). Values are means ± standard deviations. * different from HST1 ($P < 0.05$).

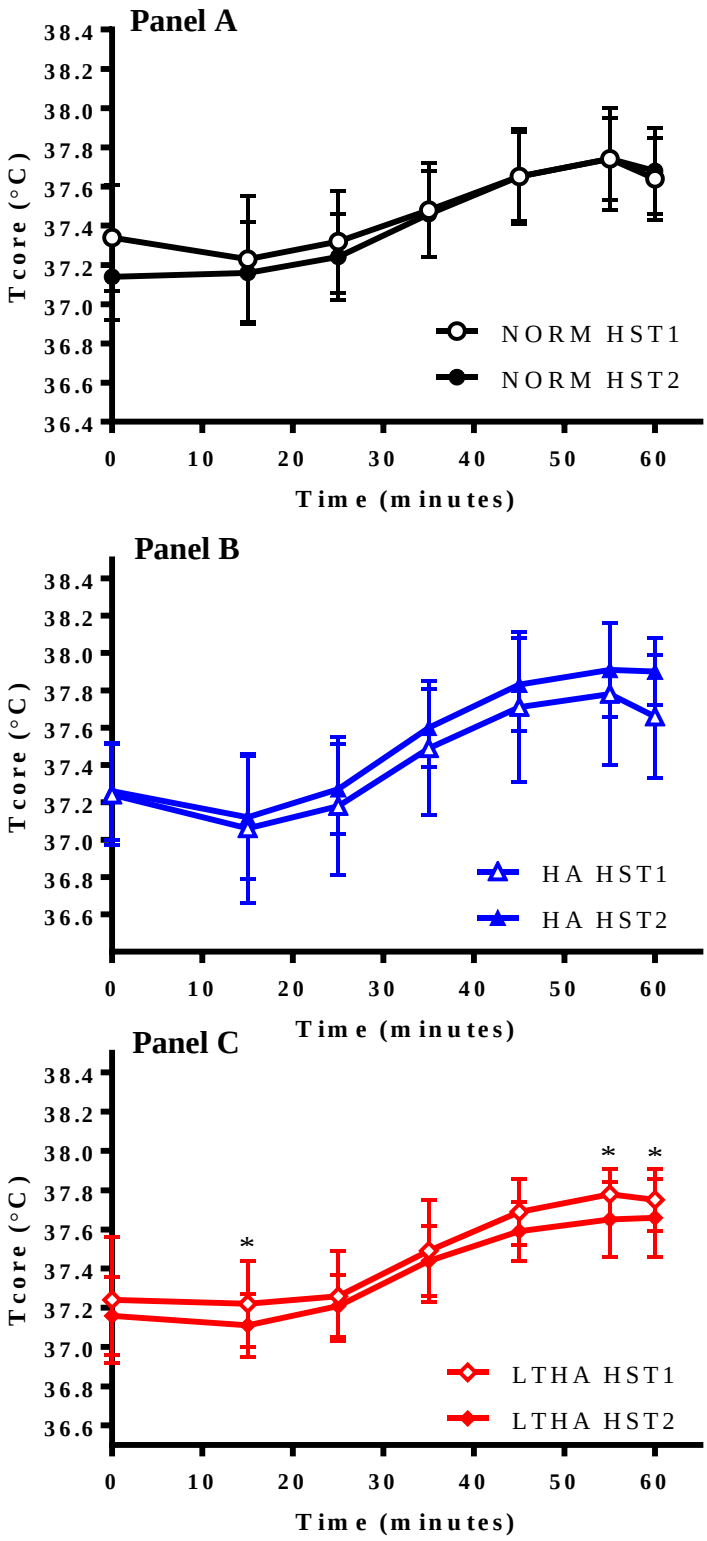

Figure 6.4. Mean ± SD T_{core} throughout HST1 (clear marker) and HST (solid marker) for NORM (Panel A, *n* = 7), HA (Panel B, *n* = 7) and LTHA (Panel C, *n* = 7). * different from HST1 ($P < 0.05$).

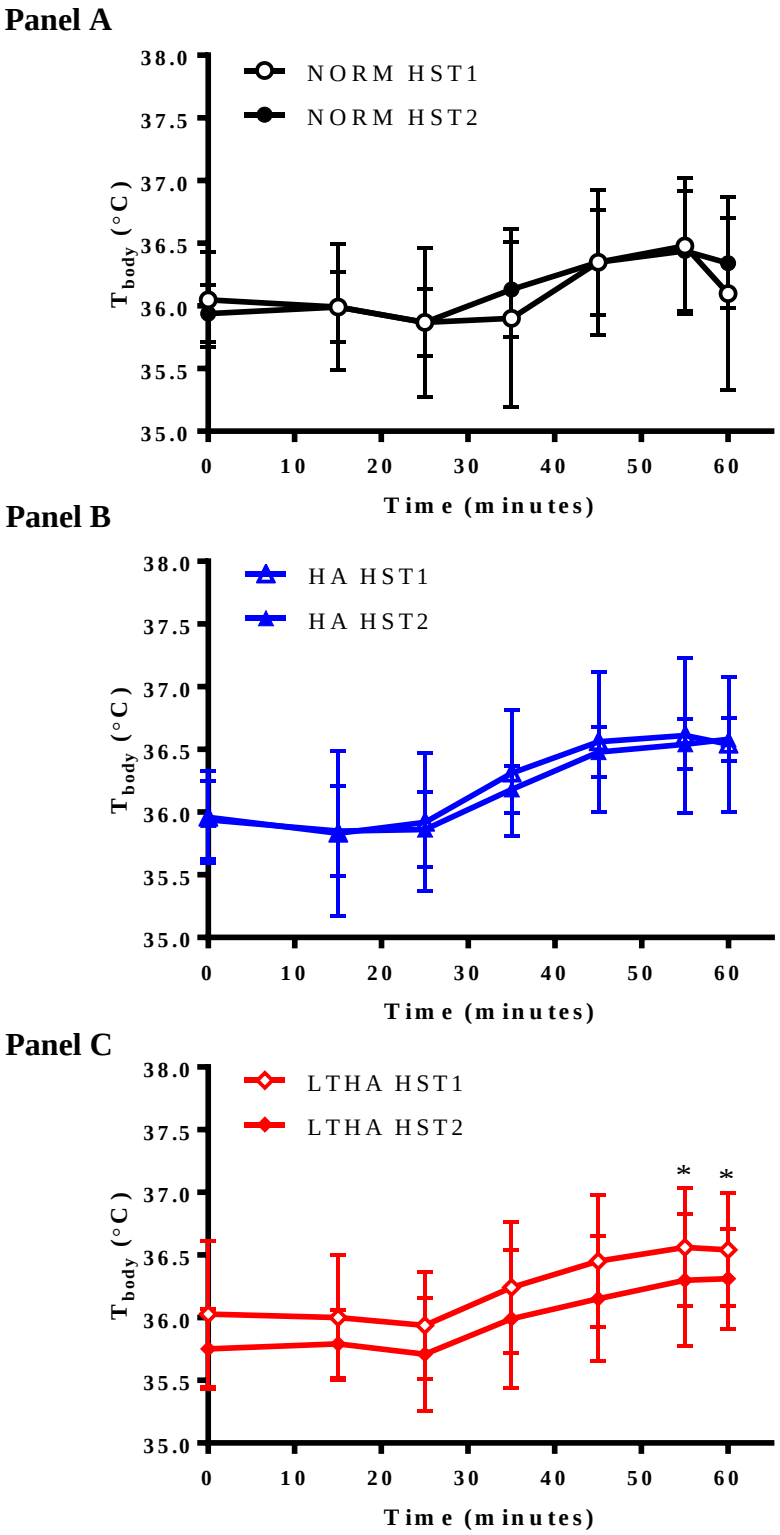

Figure 6.5. Mean ± SD T_{body} throughout HST1 (clear marker) and HST (solid marker) for NORM (Panel A, *n* = 7), HA (Panel B, *n* = 7) and LTHA (Panel C, *n* = 7). * different from HST1 ($P < 0.05$).

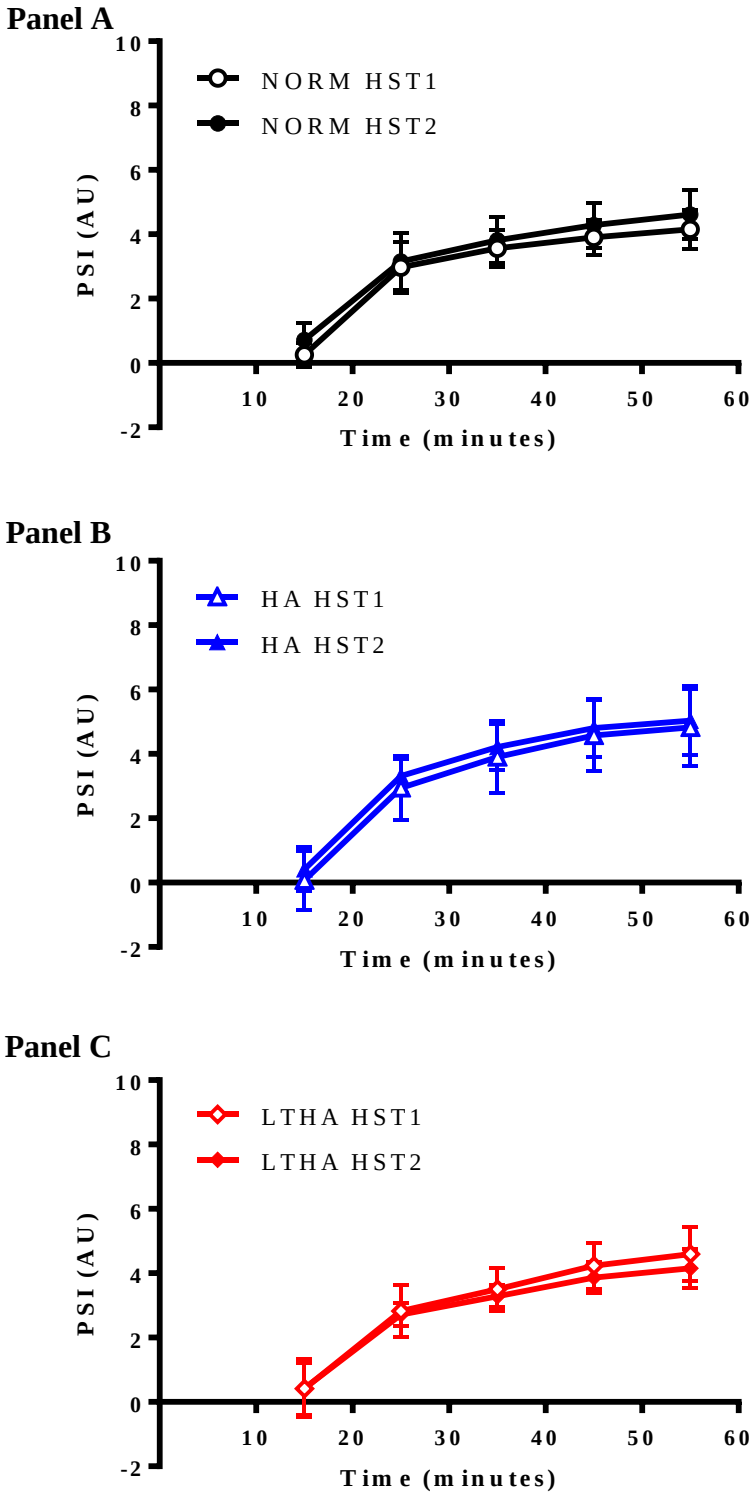

Figure 6.6. Mean ± SD PSI throughout HST1 (clear marker) and HST (solid marker) for NORM (Panel A, *n* = 7), HA (Panel B, *n* = 7) and LTHA (Panel C, *n* = 7)

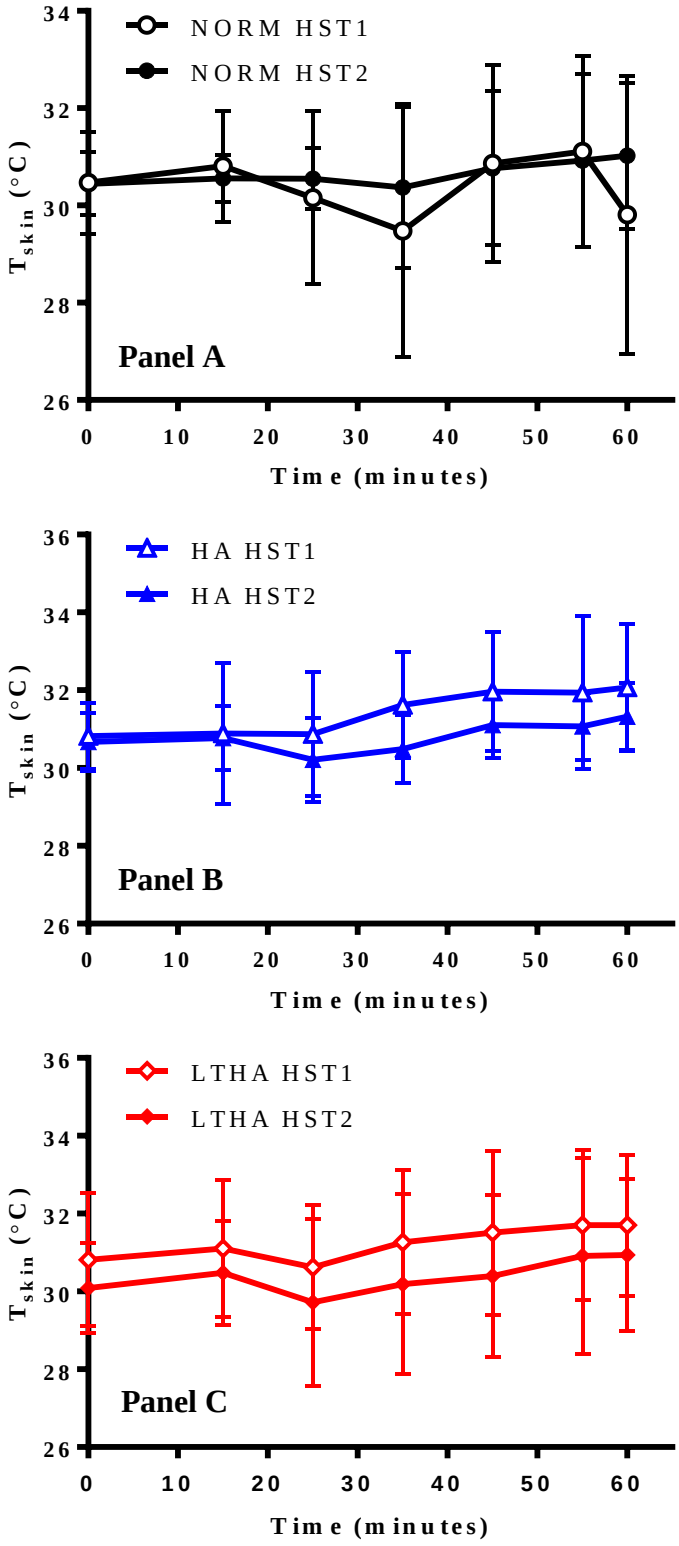

Figure 6.7. Mean ± SD T_{skin} throughout HST1 (clear marker) and HST (solid marker) for NORM (Panel A, *n* = 7), HA (Panel B, *n* = 7) and LTHA (Panel C, *n* = 7).

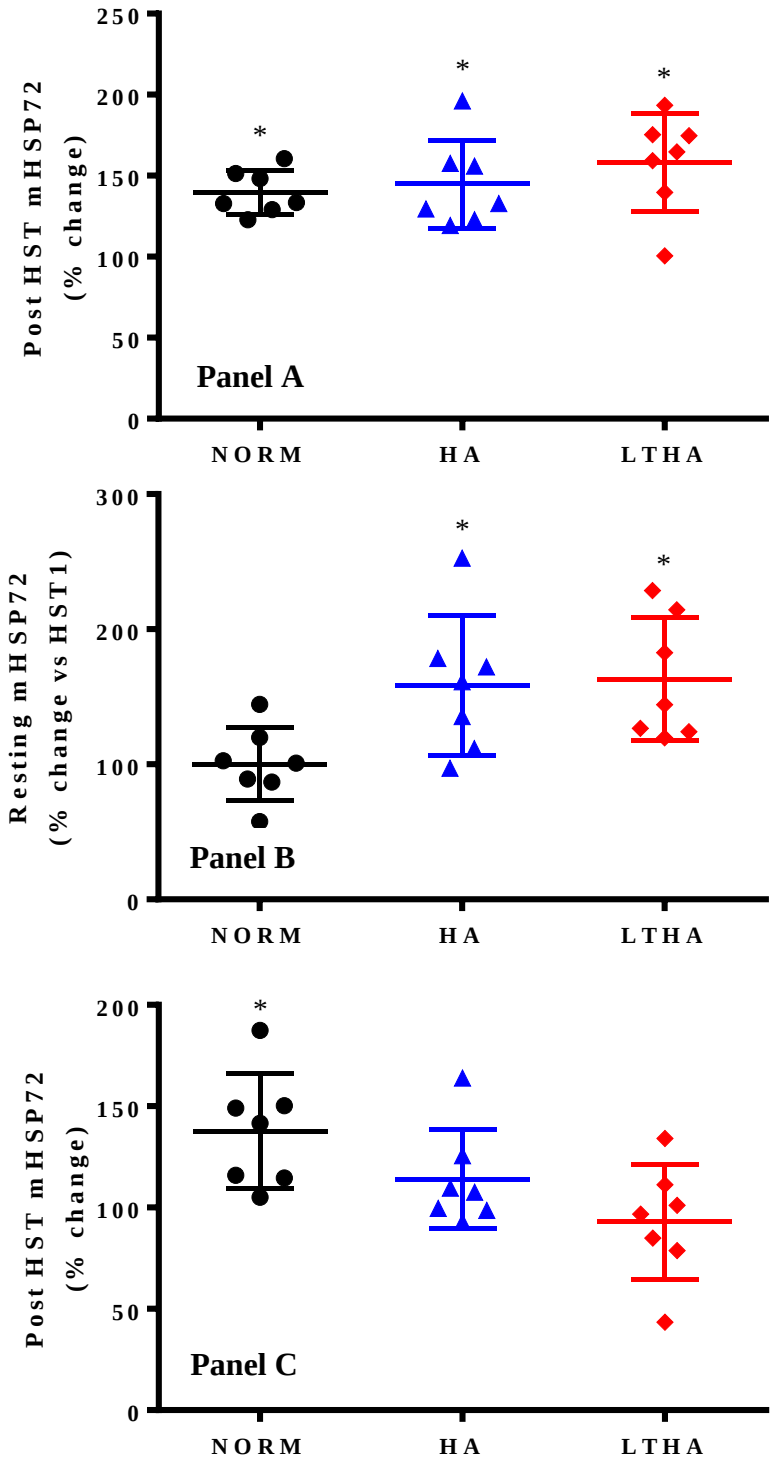

Figure 6.8. Panel A shows the fold change in mHSP72 from rest after HST1 for NORM (n = 7), HA (n = 7), and LTHA (n = 7). Panel B shows the fold change in basal mHSP72 at rest prior to HST2 relative to HST1, and Panel C shows the fold change in mHSP72 post HST2 relative to baseline mHSP72 before HST2.

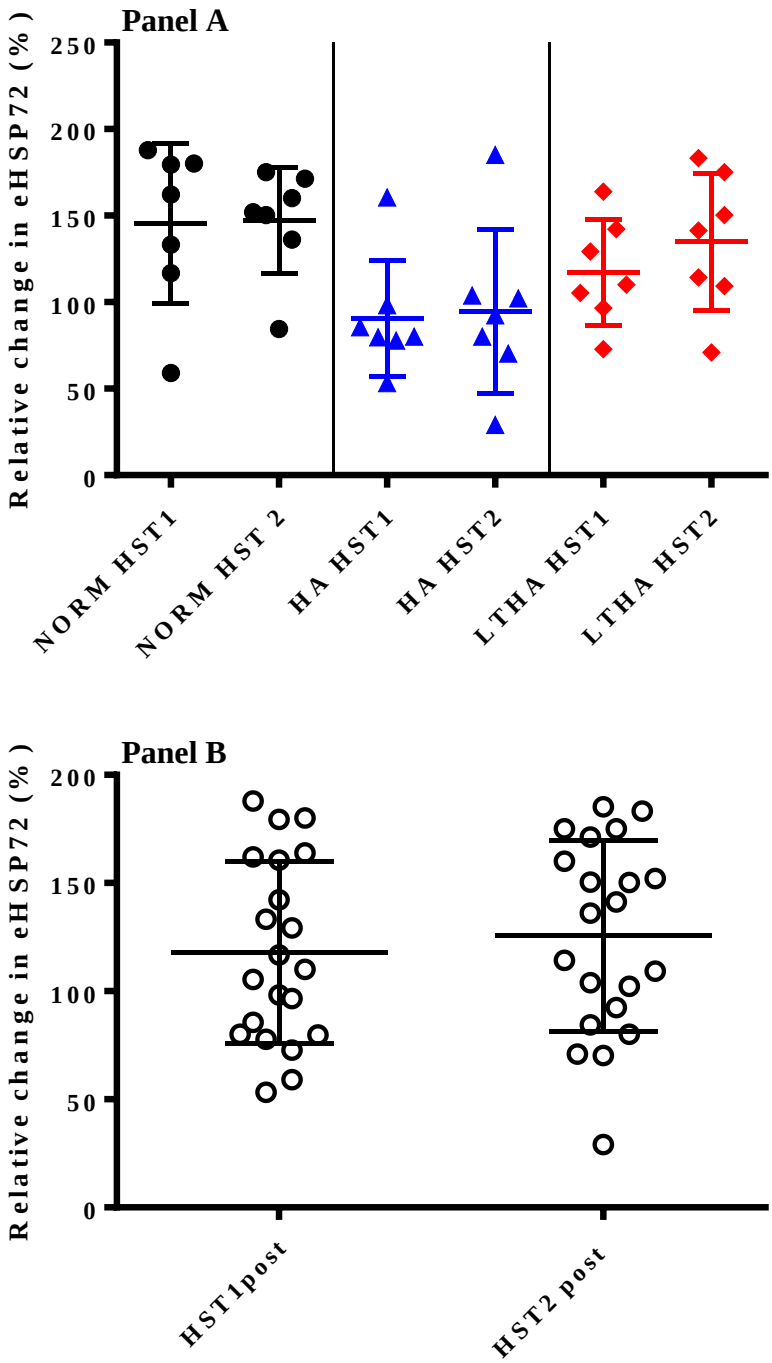

Figure 6.9. Panel A shows the fold change in eHSP72 from rest after HST1 and HST2 each individual participant in the 3 experimental groups. Panel B shows the pooled post exercise eHSP72 response for each participant following HST1 (n = 20) and HST2 (n = 21). Horizontal lines represent the mean ± SD.

Table 6.4. Mean and peak (mean ± SD) exercise data for cardiorespiratory variables during HST1 and HST2 for each experimental group.

	$\dot{V}_E$ (L•min^{-1} BTPS)		$\dot{V}O_2$ (L•min^{-1} STPD)		$\dot{V}CO_2$ (L•min^{-1} STPD)		RER		Cardiac output (L•min^{-1})		Stroke volume (mL•beat^{-1})	
	Mean	Peak	Mean	Peak	Mean	Peak	Mean	Peak	Mean	Peak	Mean	Peak
CON												
HST1	60.7 ± 13.9	61.5 ± 14.6	1.79 ± 0.43	1.85 ± 0.47	1.80 ± 0.37	1.82 ± 0.39	0.98 ± 0.10	1.02 ± 0.08	12.9 ± 1.5	14.9 ± 2.0	98 ± 14	107 ± 14
HST2	57.7 ± 16.6	59.2 ± 15.3	1.68 + 0.49	1.68 ± 0.60	1.64 ± 0.53	1.73 + 0.44	0.98 ± 0.10	1.05 ± 0.14	11.5 ± 2.3	12.6 ± 2.1	93 ± 16	91 ± 19
LTHA												
HST1	60.1 ± 8.3	60.8 ± 5.0	1.53 ± 0.13	1.76± 0.13	1.76 ± 0.14	1.78 ± 0.12	1.03 ± 0.05	1.10 ± 0.07	11.9 ± 1.2	13.7 ± 1.4	91 ± 11	98 ± 10
HST2	58.4 ± 3.8	58.8 ± 3.1	1.59 ± 0.13	1.75 ± 0.24	1.70 ± 0.16	1.78 ± 0.12	1.01 ± 0.03	1.15 ± 0.18	11.2 ± 1.5	12.8 ± 1.7	86 ± 15	92 ± 14
HA												
HST1	63.2 ± 10.1	64.6± 9.9	1.71 ± 0.26	1.74 ± 0.47	1.85 ± 0.27	1.86± 0.23	1.09 ± 0.06	1.06 ± 0.09	14.1 ± 1.5	16.1 ± 1.8	103 ± 15	111 ± 18
HST2	63.9 ± 9.6	66.0 ± 9.2	1.62 ± 0.51	1.69 ± 0.25	1.66 ± 0.52	1.82 ± 0.26	1.09 ± 0.07	1.03 ± 0.08	13.2 ± 0.9	14.6 ± 1.3	99 ± 13	105 ± 13

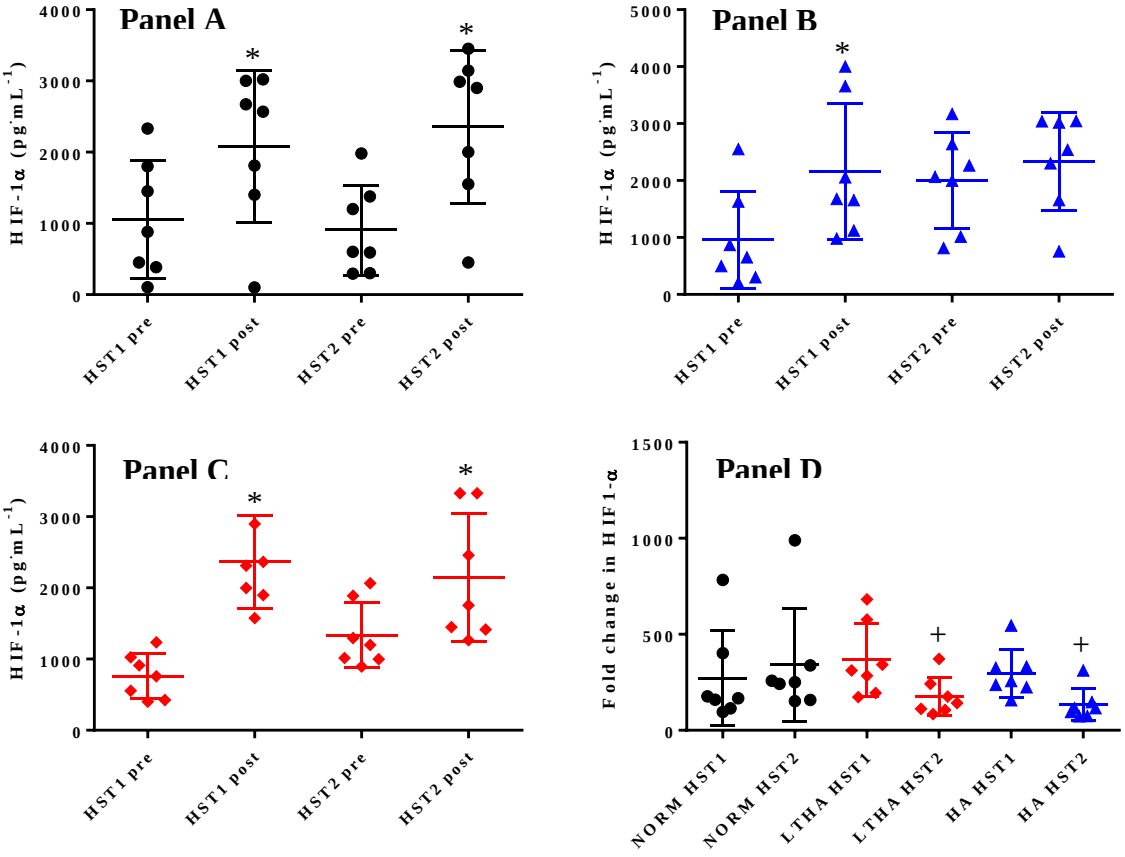

Figure 6.10. Individual plasma HIF-1α responses to HST1 and HST2 for NORM (Panel A, n = 7), HA (Panel B, n = 7) and LTHA (Panel C, n = 7). Panel D shows the fold change in HIF-1α relative to the pre-trial HIF-1. HST1 induced significant, highly variable, increases in HIF-1α in each experimental group. Following HA and LTHA, post HST2 responses were attenuated. * < 0.05 compared to rest. + < 0.05 versus HST1.

6.3.6 Hypoxic time trial performance

There was a main effect for trial (F = 11.802, P = 0.003) and a trial x group interaction (F = 3.546, P = 0.04) for time trial completion times. Time to complete the TT was not different between TT1 and TT2 in the CON group (P > 0.05). Both the LTHA and HA groups completed TT2 faster that TT1 (P < 0.05), with no difference observed between these two groups. Individual completion times for each time trial are presented in Figure 6.10 in the

upper panel. The lower panel plots the individual data against the line of no difference (before and after the 10 day intervention period) for TT performance. It should be noted that the completion time during TT2 consistently falls below this line in LTHA and HA groups, whereas 5 of the 7 data points fall on or over the line in the CON group. The LTHA and HA group show a clear and consistent performance improvement during the second TT.

Accordingly, power output during the time trial was increased in TT2 compared to TT1 in the LTHA and HA groups. Specifically, PO was greater at 1-8 and 14 – 16km in the LTHA group, and during 1 – 16 in the HA group (Figure 6.11). Heart rate and T_{core} was unchanged between TT1 and TT2 in all groups ($P > 0.05$), however physiological strain was higher in TT2 compared to TT1 in the CON ($F = 9.580$, $P = 0.02$) and HA ($F = 6.934$, $P = 0.03$) groups. Specifically, PSI was increased from 3 – 16km in CON, and 5 – 16 in HYP. Heart rate, T_{core}, and PSI data are shown in Figures 6.12 and 6.13.

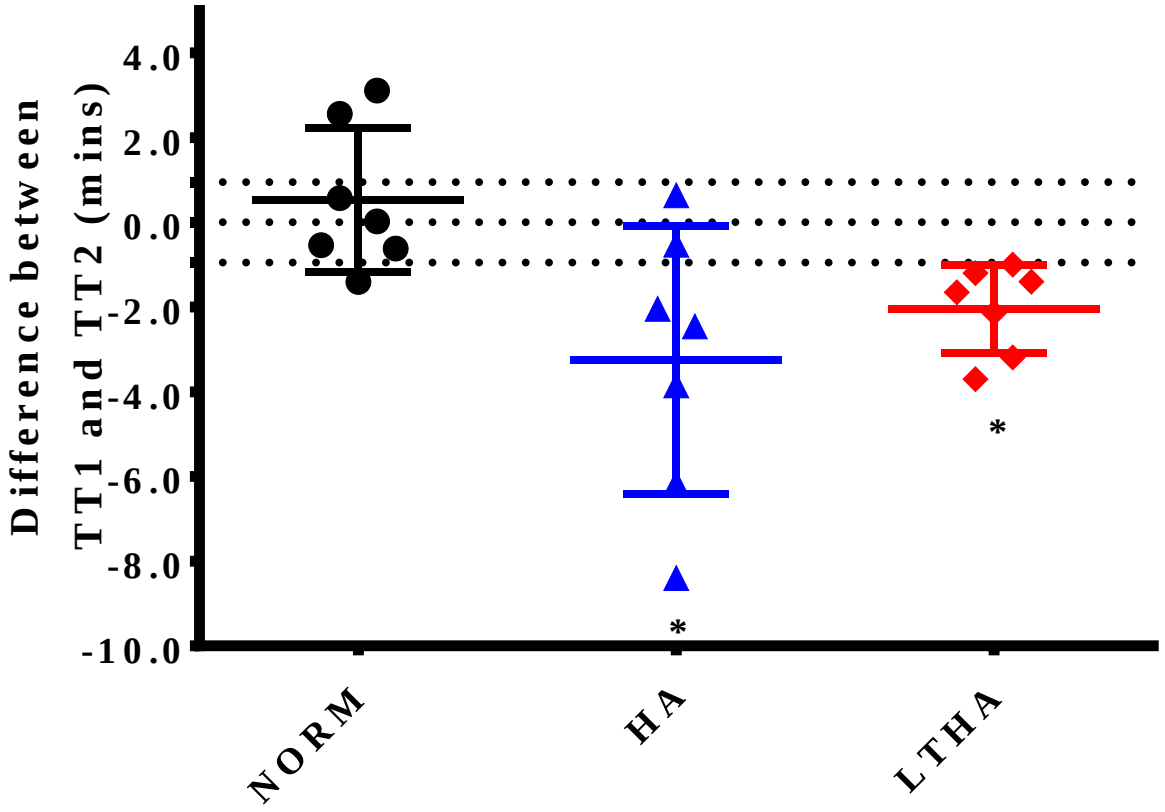

Figure 6.11. Time difference between TT1 and TT2 for NORM (black circles, n= 7), HA (blue triangles, n = 7) and LTHA (red diamonds, n = 7). The dotted lines represent the technical error of the TT (46 seconds, See section 3.11.3). Data points outside of this area represent participants that experienced a physiologically worthwhile alteration in TT performance post intervention (* $P < 0.05$).

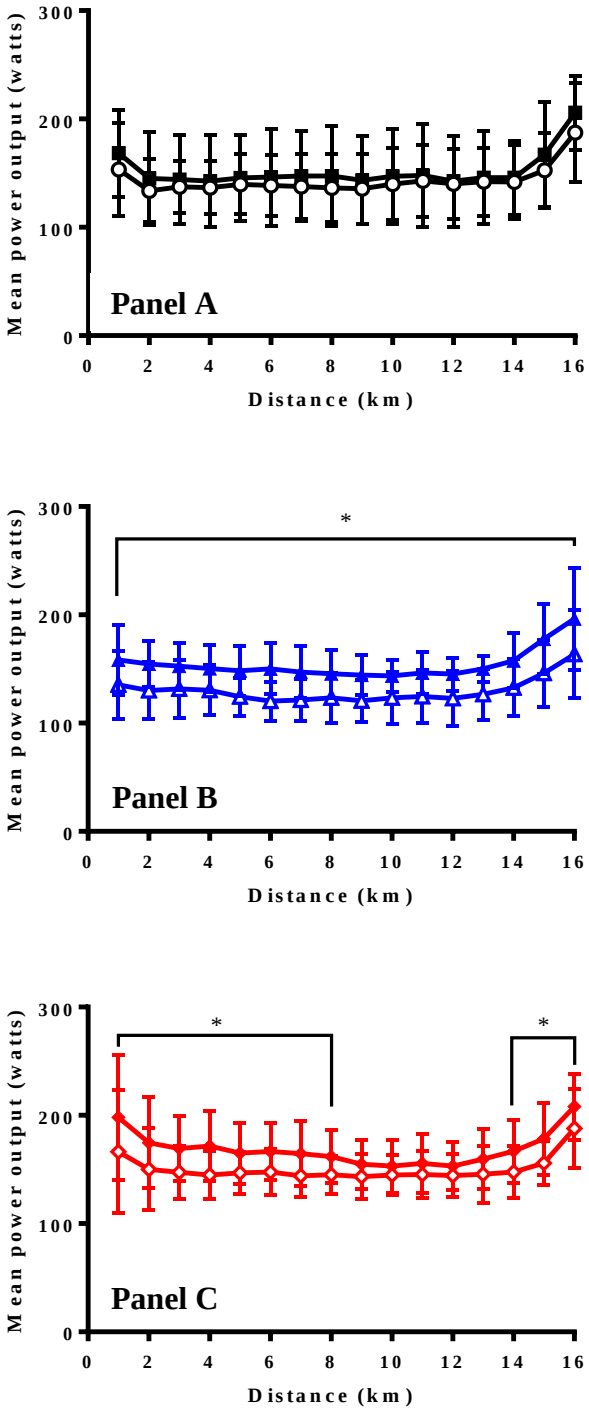

Figure 6.12. Mean ± SD power output averaged over each kilometre for NORM (Panel A, *n* = 7), HA (Panel B, *n* =7) and LTHA (Panel C, *n* = 7). * $P < 0.05$ compared to TT1.

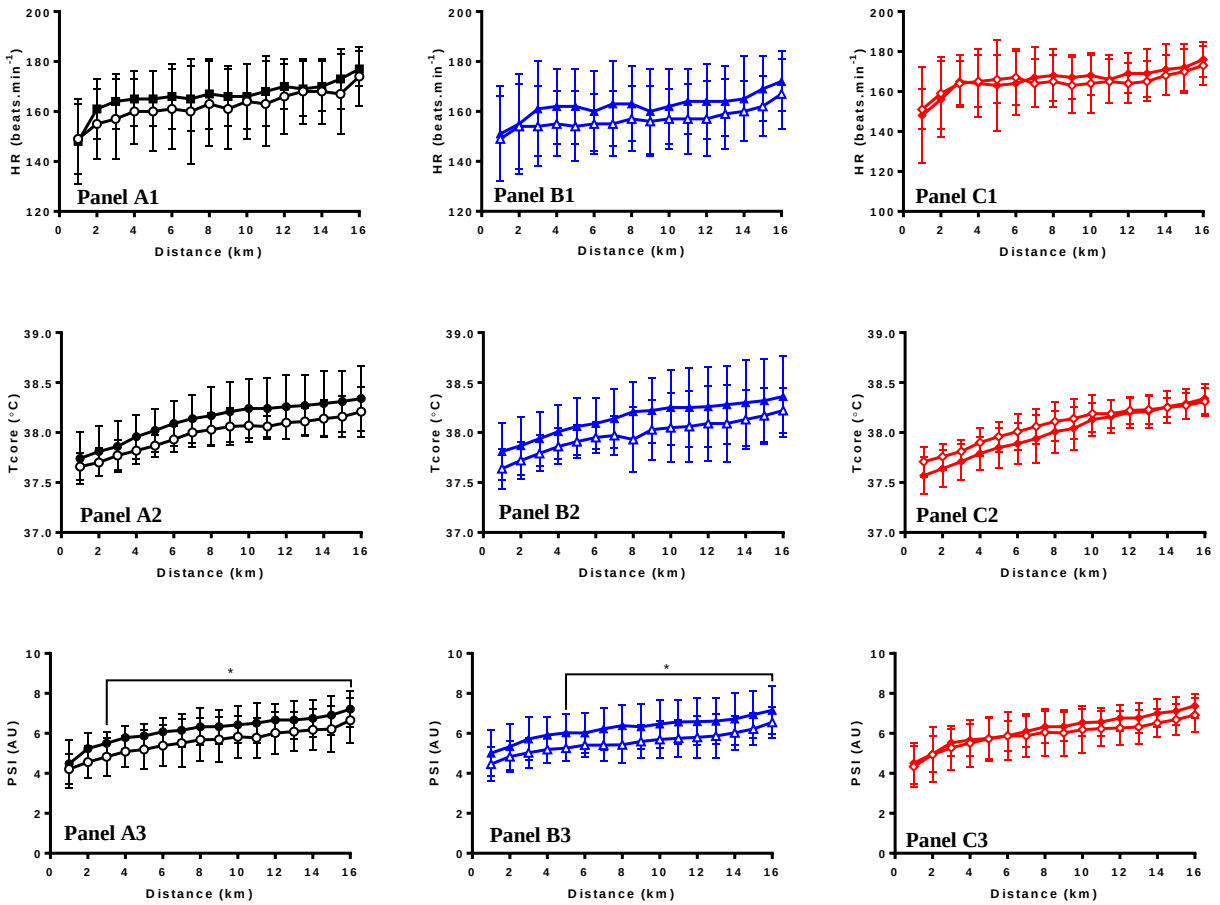

Figure 6.13. Mean ± SD heart rate, T_{core}, and PS1 averaged over each kilometre for NORM (Panels A1 – A3, *n* = 7), HA (Panel B1 – B3, *n* =7) and LTHA (Panel C1 – C3, *n* = 7). * P < 0.05 compared to TT1.

6.4 Discussion

The present study compared the effects of acclimation to either heat or hypoxia on monocyte HSP72 expression during the acclimation period, and how any acclimation mediated changes in mHSP72 affected the subsequent cellular stress response. This study is the first to examine the impact heat acclimation has on hypoxic performance, and the first to quantify HSP72 expression throughout both a heat and hypoxic acclimation period. The major findings were (1) an increase in basal mHSP72 following both LTHA and HA. This increase was more pronounced in the LTHA group, agreeing with the first experimental hypothesis (2) the hypoxia induced increase in mHSP72 following HST2 was blunted in comparison to HST1 in both the LTHA and HA, with no change observed in CON, agreeing with the second experimental hypothesis (3) plasma HIF-1α is detectable in human plasma, though is highly variable between participants. Basal HIF-1α increased as a result of HA (experimental hypothesis 3 accepted). Interestingly. LTHA also appeared to upregulate the amount of circulating HIF-1α in plasma (4) hypoxic cycling time trial performance was significantly improved in both the LTHA and HA acclimated groups compared to the CON group, with no performance differences observed between these groups, agreeing with the forth experimental hypothesis.

6.4.1 Attainment of acclimation

In this study, 10 days of fixed work exercise in either hot, hypoxic or control conditions was used to induce environmental acclimation in the participants. The classic adaptations to heat acclimation were observed, i.e. reduced resting and exercising core temperature and heart rate, an increased sweat rate, and expanded plasma volume (Garrett, 2011). In the hypoxic group, exercising heart rate was reduced and both resting and exercise minute ventilation

elevated on day 10 compared to day 1. No significant physiological changes were observed in the control group. These data show that the acclimation protocols used were effective in inducing heat and hypoxic acclimation (Millet et al., 2010; Castle et al., 2011).

6.4.2 Intracellular mHSP72 responses to acclimation

In chapter 4 it was shown that acute heat stress induced greater post exercise increases in mHSP72 compared to hypoxia and was maintained 24 hours later. The present study demonstrates similar post exercise mHSP72 responses to heat (166 ± 42%) and hypoxia (153 ± 44%) on day 1 of the acclimation period, which could be considered as an acute stress exposure. The immediate response in hypoxia is in contrast to previous work, which failed to observe significant increases in mHSP72 following a resting hypoxic exposure (75mins, 2980m) until at least 30 minutes post test (Taylor et al., 2010; 2011; 2012). This may suggest that an exercise bout induces a more rapid mHSP72 response than observed while resting. However the magnitude of the HSP72 response was lower in this present study compared to expression values seen post resting hypoxic exposure.

The differences in magnitude of response observed in exercising trials within this thesis and resting trials (Taylor et al., 2010; 2011) may be explained by the technical limitations imposed by the antibody. The antibody clone used within this thesis and in other studies (Taylor et al., 2010; 2011) recognizes only "bio-available" HSP72 i.e. unbound HSP72 not performing chaperonic functions. The binding of HSP72 to denatured proteins renders the antibody-binding site blocked and unrecognizable. The antibody clone used (SPA-810) is directed against an amino-acid sequence (aa437-50) that is within the peptide-binding domain of HSP72 (aa383–508). This has implications in all measurements of HSP72 in live cells, and especially those projects that use a stressful intervention, whereby if the stress response is initiated HSP72 maybe undetectable if performing chaperonic functions. Theoretically, and

exercising stress exposure in this present investigation would induce a greater and more immediate requirement for the chaperone function of HSP72 than a resting hypoxic exposure, and thus would be expected to induce greater levels of expression. However, if the induced HSPs are sequestered for chaperonic function early in the exercise bout they will not be detected, thus the lower levels of HSP72 observed in this investigation compared to others using resting exposures (Taylor et al., 2010). It is possible that accurate values of stress induced HSP72 may be currently unobtainable. Future work could examine the time periods during and immediately post exercise in order to determine the threshold time required to detect the peak in post stressor mHSP72 expression, which may occur at different rates following different stressors and different work intensities. This would have important implications when considering the timing of preconditioning interventions.

In the present investigation it can be seen that 10 daily exercise heat or hypoxic exposures induced significant elevations in basal mHSP72 (Figure 6.1). Interestingly, the elevation in mHSP72 demonstrated a rapid rise in the HEAT group, increasing ~15% daily until day 5 of acclimation. Extrapolating from the data, a similar daily increase would be observed between days 6 – 10. The mHSP72 expression kinetics seen during other heat acclimation protocols whereby daily hyperthermic exercise stress elicits sustained increases in mHSP72, compared to baseline levels (Yamada et al., 2007; Amorim et al., 2008; McClung et al., 2008; Magalhães et al., 2010), mirror those seen here (Figure 6.1). In contrast, the alterations in basal mHSP72 following daily hypoxic exposure appear to occur at a much slower rate, and plateauing between days 5 and 6, increasing a further ~5% daily until day 10. In totality, the heat acclimation protocol induced an increase in basal mHSP72 of ~171 ± 44% compared to the 155 ± 48% increase observed at the end of the hypoxic acclimation period. This data may

suggest that repeated daily exposure to a fixed work bout of heat stress offers a more potent stimulus for mHSP72 induction and cellular adaptation than daily hypoxia.

Furthermore, as baseline HSP72 increased throughout the acclimation period, the exercise induced increase in HSP72 was not required from day 3 in the LTHA group (Figure 6.1). These responses are in agreement with recent studies on intracellular HSP72 in heat acclimation in humans and animals, and the limited research utilizing hypoxia (Yamada et al., 2007; McClung et al., 2008; Kuennen et al., 2010; Taylor et al., 2010; Taylor et al., 2010; Taylor et al., 2011). Maloyan (1999) showed that passive heat acclimation increases basal expression of HSP72 in rat cardiac muscle and reduces *in vitro* heat shock increases in HSP72. Marshall et al., (2007) observed no changes in post exercise HSP72 expression following 2 days of heat acclimation. However, although some physiological changes were noted, the exercise intensity (~42% $\dot{V}O_{2\ peak}$) and peak T_{core} (38.15°C) achieved may not have been enough to induce acclimation. In Chapter 5, 3 days of heat acclimation was found to induce increases in basal mHSP72, and attenuated post exercise heat stress mHSP72 following the third day of acclimation. McClung et al., (2008) observed an increase in HSP72 following 10 days of acclimation. These authors also observed that when cells from acclimated participants were given an *in-vitro* heat shock, there was a blunted HSP72 increase compared to that seen prior to acclimation. The results of the present investigation support those of Yamada et al., (2007) and McClung et al., (2008), showing that heat acclimation increases basal HSP72 in humans. This study also shows that hypoxic acclimation induces increases mHSP72, supporting Taylor et al., (2011; 2012) and that when baseline levels of mHSP72 are elevated, the stress induced increase in HSP72 is blunted *in-vivo*. The mechanism by which increased baseline HSP72 may inhibit its own expression is related to HSP72 binding to heat shock factor 1 (HSF-1). HSF-1 is inactive in unstressed cells

and is present as a homodimer bound to HSP72. Stress conditions require that HSP72 bind to denatured proteins, thereby freeing HSF-1, which trimerizes, migrates to the nucleus and binds to the heat shock element. This increases HSP72 levels, binding with HSF-1 and inhibiting its own transcription (Morimoto, 1998). Conceptually, following an acclimation mediated increase in mHSP72, the stress required to induce HSF-1 activation would need to be higher, with the results of the present study supporting this hypothesis.

The constant work rate acclimation protocol used in this study reduces the degree of physiological strain, and therefore cellular strain, that participants experience as a result of a shift towards the acclimated phenotype. These adaptive responses also serve to diminish the requirement for additional HSP72 induction. That baseline HSP72 failed to increase above the post exercise levels attained on day 1 of acclimation following 9 further exposures in both experimental groups in this investigation is an interesting observation. Magalhaes et al., (2010) used an isothermic model of acclimation, in which daily physiological strain was maintained throughout 11 days of acclimation by adjusting the work rate where necessary. These authors reported a 4-fold increase in basal HSP72 following the acclimation period, and a significant attenuation of HSP72 following a final heat stress test. Basal levels post acclimation were increased beyond those seen post pre-acclimation heat stress test. This would suggest that a continual level of maintained daily physiological strain acts as a stimulus for greater HSP72 induction and may induce a more complete level of physiological and cellular acclimation.

6.4.3 Hypoxic tolerance and time trial performance

Adaptation to hypoxic conditions requires a gradual and often lengthy exposure to hypoxia. Thus it is practically important to find a method that may improve tolerance to hypoxia and improve performance. The hypothesis of this study was that as adaptation to heat and hypoxia

share several molecular and metabolic mechanisms, a cross adaptation between these two stressors maybe expressed in whole body physiological performance and cellular adaptation. The purpose of the study was to directly compare a period of normobaric hypoxic acclimation with a period of heat acclimation in similar participant cohorts to see whether heat acclimation may serve as a preconditioning method that preserves essential physiological and cellular functions upon later normobaric hypoxic exposure.

The findings of this study add to those described in Chapter 5, in that a period of heat acclimation may be associated with the improvement of both physiological and cellular tolerance during later exposure to mild hypoxic conditions. A surprising finding in the current study was that 10 days of normobaric hypoxic acclimation induced no physiological improvements in tolerance during the second HST compared to the first. This occurred despite clear reductions in exercising heart rate on day 10 of hypoxic acclimation compared to day 1 (Table 6.2). During HST2, heart rate was reduced by between 9 and 10 beats•min^{-1} at 30 and 40 minutes of hypoxic exercise in the LTHA group ($P < 0.05$). In contrast no differences were observed in the HA group. Reductions were also seen in exercising T_{core} and T_{body}, alongside increases in sweat rate. These adaptations are characteristic of the heat acclimated phenotype and may have preserved cardiac efficiency during the hypoxic challenge. The LTHA group also had an expanded plasma volume prior to HST2 in contrast to the contraction of plasma volume seen in the HA group. Plasma volume expansion has been largely attributed to the increased cardiac stability observed following periods of acclimation (Garrett et al., 2011), thus it is not inconceivable that heat-acclimation mediated hypervolemia would also improve cardiac stability during a period of brief moderate hypoxic exercise.

Heat acclimation has been shown to improve physiological performance under sea level normothermic conditions, as a result of and improved aerobic capacity and positive effects on lactate thresholds (Lorenzo et al., 2010; 2011). That participants in either the LTHA or HA group did not improve their aerobic profile (Table 6.3) as a result of the acclimation period may strengthen conclusions regarding the effects of heat *per se* on hypoxic tolerance. This could suggest that heat, and not a training effect is the main effector of increased physiological efficiency observed in the hypoxic conditions studied herein.

Time trial performance was unaffected in the CON group, and improved in the LTHA and HA groups compared to HST1. No difference in TT performance was observed post acclimation between heat and hypoxia. This represents the first study to observe improvements in hypoxic exercise performance following a period of heat acclimation in humans. The mechanism behind the improved performance is unclear with no alterations in aerobic profile observed in either experimental group. This indicates that heat and hypoxia specific adaptations likely contributed to this modest improvement in TT completion time. The HA group had a higher heart rate in conjunction with the higher average power output throughout TT2 compared to TT1. This may suggest that participants in this group were able to work at a higher relative exercise intensity following the hypoxic acclimation period, as post acclimation aerobic profiles remained unchanged in each group (Table 6.3). That T_{core} was also higher in TT2 points to an increased metabolic rate. The increase in effort during TT2 may be partly due to the reduced perception of strain in the initial constant load aspect of the test, highlighted by the reduced mean exercise RPE (mean RPE; HST1 12.2 ± 0.9: HST2, 10.2 ± 0.5). Published findings on continuous and intermittent hypoxic acclimation generally agree with present observation of improved TT performance at 3000m after 10 days of

intermittent hypoxic exercise (Maher et al., 1974; Gleadle et al., 1997; Beidleman et al., 2003; Fulco et al., 2005; Beidleman et al., 2007; Fulco et al., 2011).

The improved TT performance in the LTHA group appears to be characterized by an altered pacing pattern during the early stages of the TT. This may be explained by reductions in RPE during the initial constant load test (HST1 12.8 ± 1.4; HST2 10.2 ± 0.3). Heart rate, T_{core} and PSI were each lower prior to the start of TT2 compared to TT2, and may explain the initial increase in work rate. RPE has been linearly correlated with T_{core} during exercise in both hot and cool conditions. Thus the lower T_{core}, and likely lower RPE during the initial stages of TT2 may have allowed for the initial increases in power output in the LTHA group (1-8km). Once T_{core} values approached those observed in TT1 at 9 – 10km, (Figure 6.9) the differences in power output between time trials diminished.

During the final stages of the time trial the "end spurt" phenomenon, in which power output increases significantly during the closing stages of self-paced trials (Tatterson et al., 2000; St-Clair Gibson et al., 2001 Marino et al., 2004; Albertus et al., 2005) was evident. The onset of the end spurt appears to begin earlier during TT2 in the heat group. These results indicate that both heat and hypoxic acclimation has the potential to improve physiological performance in moderate hypoxic conditions. It is likely that these performance improvements are moderated by different mechanisms which in the absence of an improved aerobic profile post acclimation may be attributed to altered pacing strategies linked to reductions in perceived exertion either before or during the TT. The reduced T_{core} at the start of the TT in the LTHA group may have allowed participants to work harder until a critical level of T_{core} and therefore RPE was attained, leading to the mid TT reductions in power output seen in this group. It would be useful to examine whether the magnitude or direction of performance is altered when the initial constant workload phase of the trial is omitted.

6.4.4 Intracellular mHSP72 responses to the HST

Current available evidence indicates that a reprogramming of gene expression and translational processes are essential for acclimation (Maloyan et al., 1999; Maloyan et al., 2005; Horowitz et al., 2007). One of the hallmarks of cross acclimation identified in rodent models of both heat acclimation and heat acclimation mediated cross tolerance include enhanced reserves of HSP72 (Tetievsky et al., 2008). This present investigation provides initial tentative evidence that both heat acclimation and hypoxic acclimation enhances the HSP cytoprotective network. This study provides the first evidence that a 10-day period of daily 1-hour heat or hypoxic exercise acclimation induces similar alterations in pre and post exercise mHSP72 expression kinetics, which are maintained up to 48 hours after the final acclimation stress. This shared cellular adaptive mechanism could therefore point to a mechanism that induces cross acclimation between these stressors in human participants. Both LTHA and HA acclimation induced a larger steady state stock of mHSP72 prior to HST2 (Figure 6.3). Resting mHSP72 expression prior to HST2 were similar to the mHSP72 expression observed *after* HST1. Therefore it is not surprising that no change in mHSP72 was observed in either the LTHA or HA groups after HST2. Acclimation in each group would have increased the induction threshold stress that would be required to raise post exercise mHSP72. It may be suggested that the acclimated participants would be able to tolerate a more severe level of hypoxia in order to generate the same level of cellular strain encountered in HST1, e.g. a 25% post exercise increase in mHSP72.

These results support those seen in Chapter 5, where the short-term heat acclimation mediated increases in basal mHSP72 attenuated the mHSP72 response to hypoxic exercise. A strength of this present study is the inclusion of a hypoxic acclimation group which allows direct comparison between LTHA and HA, an experimental arm not included in Chapter 5

nor previous research study examining cross acclimation in humans (Heled et al, 2012). The increased basal HSP72 expression after acclimation to each stressor allows a faster cellular response to disruptions in cellular homeostasis. That each stressor induces the HSR response following an acute exposure (Fehrenbach et al., 2005; Taylor et al., 2010) would suggest that this is a shared cellular stress response, and therefore also a shared adaptive pathway that requires further study in humans. The discovery of quercitin as a heat shock transcription factor blocker in humans allows the role of HSP72 in both acclimation and cross acclimation to be studied further. Kuennen et al., (2010) found that the typical physiological adaptations to heat acclimation were impaired following a period of quercitin supplementation, which impaired the heat shock response. A similar approach could be taken in future cross acclimation studies in order to determine the biological significance of altered basal HSP72 and the attenuated post stressor response following acclimation.

6.4.5 Extracellular HSP72 responses to the HST

In contrast to the data in chapter 5, the initial HST in this present study failed to induce an increase in eHSP72 (Figure 6.4). The duration in which individuals are in a state of physiological stress may be a contributing factor towards increasing eHSP72 concentrations (Gibson et al., 2013), thus the shorter period of hypoxic exposure in this present study (55 minutes versus 75 minutes) may partially explain this result. It is known that training status influences the resting and exercise induced stress response of eHSP72. Prior induction of a heat acclimation phenotype, or progress towards this state via endurance training has been shown to increase the immune system response threshold for eHSP72 induction (Njemini et al., 2004). The threshold for an enhanced eHSP72 release, endotoxin leakage and inflammatory activation in response to high external temperatures has been shown to occur at a lower threshold stress in untrained versus trained individuals (Selkirk et al., 2008; 2009).

Thus the individual and changing threshold along a continuum modulated by a large range of factors suggest that a reliable and consistent response of eHSP72 may be difficult to obtain as interventions may fail to sufficiently stress some individuals. Studies which thoroughly investigate the eHSP72 response to acute bouts of hypoxia for different durations of exercise and levels of altitude would be useful in determining the minimal endogenous criteria required to elicit eHSP72 release under hypoxic conditions. Heat acclimation reduced the levels of basal eHSP72 whereas hypoxic acclimation had no affect on eHSP72. This has been previously shown in heat acclimation research (Krefelder et al., 2005; Marshal et al., 2006; Sandstrom et al., 2008; Magalhaes et al., 2010), though the reason for this decrease in resting eHSP72 and attenuation of post exercise eHSP72 release is not currently clear.

The role of eHSP72 release following exercise is currently not known. It has been suggested to contribute to exercise-related inflammatory reactions, though these mainly occur after prolonged, intensive exercise (Asea et al., 2003) and not following the short bout of exercise performed in the present investigation. Post exercise increases in eHSP72 have been proposed to have immunological functions (Campisi et al., 2003), and act as a signal for cytokine and inflammatory pathways in response to unaccustomed systemic or whole body stress (Asea et al., 2000). It could be argued that until the precise role of eHSP72 has been identified, its measurement in human exercise studies reveals little about the whole body physiological processes and limits discussion to speculation on its function in relation to a given stimuli.

6.4.6 Plasma HIF-1α

This is the first study to examine the plasma HIF-1α response to hypoxic or heat acclimation in humans, using a recently developed ELISA assay (CUSABIO BIOTECH, Newark, New Jersey). Plasma HIF-1α was detectable in 19 out of the 21 participants prior to the initial

HST, and detectable in all participants 48 hours after the 10-day acclimation period. There was a considerable inter-subject variation in HIF-1α. Prior to HST1, resting values were 895 ± 589pg•ml^{-1} (n = 20). This variation was also present in normative controls (n = 26, HIF-1α = 761 ± 711pg•ml^{-1}) diabetic patients without vascular disease (n = 20, HIF-1α = 2366 ± 549pg•ml^{-1}) and diabetic patients with vascular disease (n = 20, 2448 ± 705pg•ml^{-1}) (Jiang et al., 2013). The initial HST induced a significant increase in post exercise HIF-1α although the large standard deviation was also present (Figure 6.5). Following the acclimation period, both the LTHA and HA show an increased basal level of HIF-1α, with attenuated post exercise values, though the large standard deviations observed limit the conclusions that can be drawn from this apparent trend. The results indicate further study in this area is warranted, with measurements made throughout the acclimation period. Any changes in plasma HIF-1α as a result of LTHA may have been missed due to the final sampling points occurring 48 hours after the final acclimation session. The decision to only measure this transcription factor before and after each HST was due to financial restrictions.

6.5 Conclusion

In summary, the novel findings reported in this study point to the possibility of heat acclimation being a useful conditioning tool prior to exposure to acute hypoxic conditions, for both physiological tolerance and performance purposes. The positive cellular adaptations observed in this study support this conclusion. These results are relevant to those resident or working at sea-level that are required to quickly relocate and perform at high altitude without the time to acclimatize.

Chapter 7. Discussion.

The aim of this thesis was to investigate and establish whether any *in-vivo* cellular preconditioning (PC) or cross-acclimation effect could be induced in humans between heat and hypoxic stressors. Acute (Chapter 4) and repeated (Chapter 5 and 6) daily heat exposures, which have been shown in both animal and human studies to increase the protective HSP72 protein (Maloyan et al, 1999; McClung et al., 2008), were used to examine the how prior preconditioning and longer term acclimation adaptations affected the physiological and cellular responses to acute exercise in hypoxia. The magnitude of the responses between acute heat and hypoxic exposure, and over the course of acclimation to heat and hypoxia were also compared. The purpose of this final discussion chapter is to integrate findings from the experimental studies to form an overall conclusion from the research conducted, as well as discussing the limitations and directions for future work.

7.1 Improved cellular tolerance after heat exposure

Many previous investigations have examined the intracellular HSP72 response to acute heat (Fehrenbach et al., 2003; 2005) whilst fewer studies have examined the intracellular HSP72 response to acute hypoxia (Taylor et al., 2010; 2011; 2012). Those previous studies that have investigated the HSP72 responses to hypoxia have used isolated cells (Sonna et al., 2004), animal tissue or humans exposed to a resting exposure (2095m, Taylor et al., 2010; 2011; 2012). It has proved problematic to make comparisons between previous environmental stress response studies due to the varied techniques used for HSP72 quantification (e.g. northern and western blotting, ELISA). HSP72 has been shown to play an important role in both the preconditioning and cross acclimation responses in animals (Horowitz et al., 20007), thus it was important in this thesis to address the mHSP72 response to both heat and hypoxic stress

within the same cohort of participants in order to determine the magnitude and potential each stressor has to induce any PC response. Determining the extent of the HSP72 response to these different stressors could then be refined into a protocol aimed at inducing HSP72 in order to investigate the effectiveness of cross-tolerance interventions.

The consistency of the mHSP72 response to acute hypoxia and acute heat stress was observable across each study within this thesis. When the mHSP72 response results to a time-matched acute hypoxic exercise bout were combined (Each 75 minute HST; n = 59 observations) a 132 ± 27% (95% CI = 125 – 139%) response immediately post exercise was observed (Figure 7.1, Panel A). The magnitude of the mHSP72 response to the HST was also consistently shown to be inversely proportional to the pre-trial sample following the initial HST of each study (Figure 7.1, Panel B), a feature of the HSR well reported within the heat shock literature (Vince et al., 2010).

A matched period of acute heat exercise (post acclimation day 1, Chapter 5 and Chapter 6, n = 15) induced a 161 ± 49% (95% CI = 135 – 188) post exercise increase in mHSP72 (Figure 7.2, Panel A) and also displayed the inverse relationship between resting and post heat stress mHSP72 response (Figure 7.2, Panel B). The inverse relationship between resting mHSP72 and post exercise mHSP72 induction thus appears to be shared between heat and hypoxic stressors. That each stressor appears to share such an inverse relationship between resting expression and post exercise induction lends support to the notion that both shared molecular and physiological responses are indicative of preconditioning and cross acclimation potential. These data also suggest that at the levels studied, 40°C is more time efficient stressor than 3000m asl for inducing a cellular stress response.

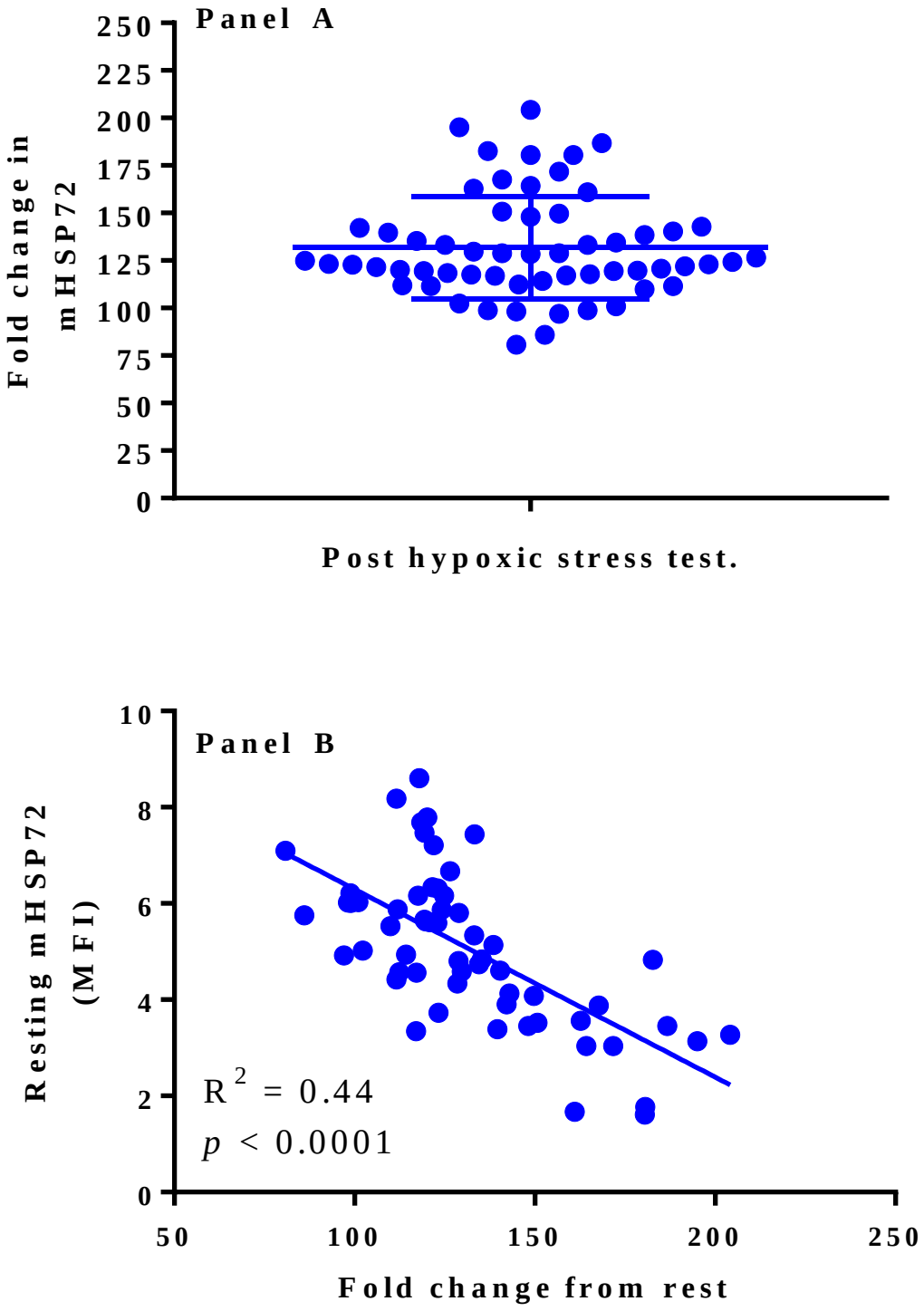

Figure 7.1. A 75 minute HST (15 minutes rest, 60 minutes cycling at 50%$\dot{V}O_2$max) induced a 132 ± 27 (95% CI, 125 – 139%) increase in mHSP72 immediately post exercise (n = 59, Panel A). The fold change in post exercise mHSP72 was inversely proportional to the resting mHSP72 MFI (Panel B).

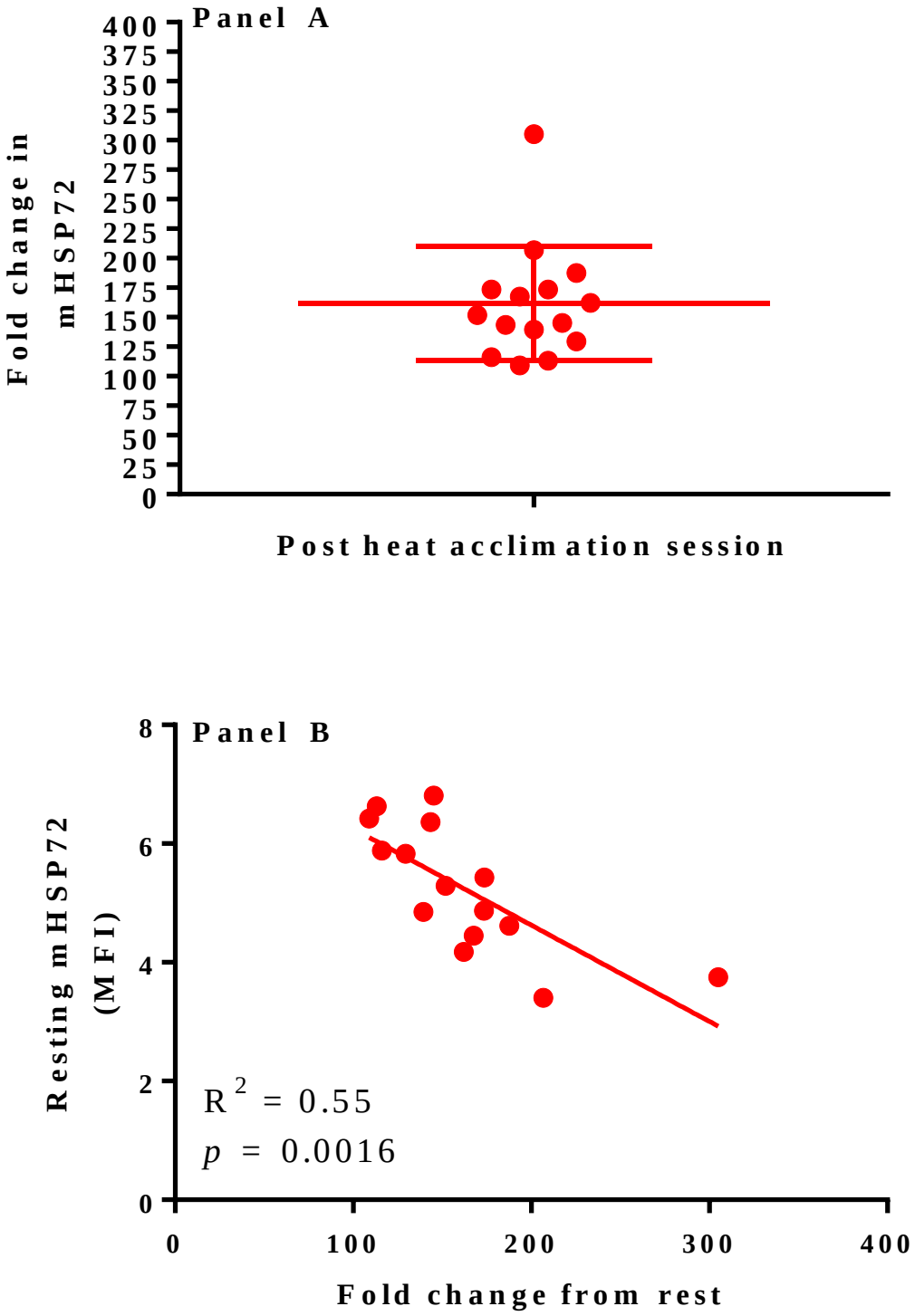

Figure 7.2. A 75 minute bout of heat stress (15 minutes rest, 60 minutes cycling at 50% $\dot{V}O_2$max) induced a 161 ± 49 (95% CI, 135 – 188%) increase in mHSP72 immediately post exercise (n = 59, Panel A). The fold change in post exercise mHSP72 was inversely proportional to the resting mHSP72 MFI (Panel B).

This thesis addressed each phase of the adaptive process, from the initial preconditioning response to an acute forcing function (90 minutes of exercise in 4 environmental conditions), the early phase of acclimation (Short term heat acclimation, STHA) and a full acclimatory cycle (long term heat acclimation, LTHA, or hypoxic acclimation, HA) on the expression kinetics of mHSP72. To summarise, it was demonstrated that acute exercise and heat stress (40°C, 20%RH) consistently induced a greater induction of mHSP72 than the same exercise conducted under hypoxic conditions (Chapter 4, 5, 6). An independent samples t-test comparing post HST (n = 59) and post HA1 (n = 15) data from all experimental chapters shows that 75 minutes of acute exercise heat stress induces a significantly greater post exercise increase in mHSP72 (t = 3.191, P = 0.002), with a mean difference of 30 ± 9% (95% CI; 11 – 49%) than observed for hypoxic stress. When effect sizes are compared, post exercise heat stress (d = 2.02; 95% CI 1.09 – 2.84) clearly induces a great mHSP72 response compared to hypoxic exercise (d = 0.99; 95% CI 0.60 – 1.37; Figure 7.3).

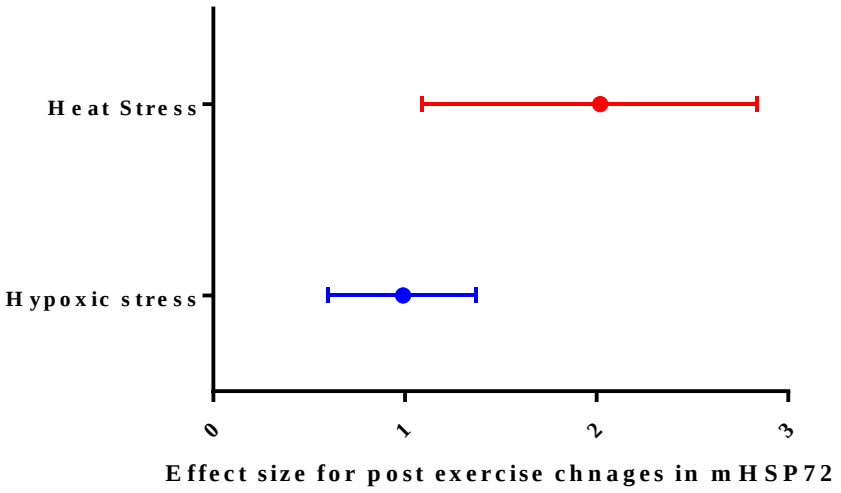

Figure 7.3. Mean ± 95% confidence intervals for effect size immediately post heat (n = 15) or post hypoxic (n = 59) exercise mHSP72 response.

The greater post exercise increase in mHSP72, perhaps unsurprisingly, led to greater elevations in mHSP72 24 hours after the initial stressor (Chapter 4), although it is unclear whether this represents an increase of basal mHSP72 or maintenance of the post-exercise elevations. A study in which a time course of the mHSP72 response is paired with mRNA data would be able to distinguish between these two possible outcomes and better delineate the optimal window of opportunity to apply the PC stressor.

Similarly, repeated daily heat exposures (3 days and 10 days) produced a cumulative increase in basal mHSP72 over the course of acclimation. However elevated basal values never exceeded the highest post exercise value achieved (usually post exercise after the initial heat acclimation session), and began to plateau once they neared this 'ceiling' value (Chapter 5, Chapter 6). Participants that achieved the greatest heat shock response following either a PC exercise bout, or period of acclimation displayed a greater attenuation in mHSP72 response post hypoxic exercise. From a physiological perspective, it appears that the added strain of exercise heat stress, (as evidenced by the higher heart rates observed in conditions of 40°C compared to 3000m asl) either acutely (Chapter 4) or repeated over multiple days (Chapter 5 and 6) induced an apparent physiological adaptation to acute normobaric hypoxic exercise, and this was more pronounced than a matched period of hypoxic exercise or acclimation. These results add support to the notion that strain *per se*, plays an important role in any adaptive process (Cotter, 2013).

7.2 The physiological relevance of improved cellular tolerance

When examining the cellular HSR in humans, or indeed any molecular responses it is reasonable to ask what degree of intracellular HSP72 accumulation (or molecular accumulation of any protein, or gene) represents a response that is physiologically meaningful and clinically relevant, and how does any observed increase affect whole body physiological function?

Determining the effect such an increase mHSP72 (and other markers of cellular stress) has on physiological function is experimentally challenging. For example, both heat and hypoxic exercise reduce visceral blood supply and induce intestinal ischemia affecting the permeability of the gut wall. As a result of this, the accumulation of circulating endotoxins and increases in plasma and intracellular cytokines have been linked to exertional heat stress (EHS; Selkirk et a., 2008, 2009) and heat stroke (Leon et al., 2012). The HSR and role of HSP72 is known to enhance epithelial barrier resistance (Doklandy et al., 2006) and reduce inflammatory cytokine production via inhibition of NF-κB (Doklandy et al, 2010). Thus it follows that strategies that induce the HSR would be beneficial in such circumstances, and those that inhibit it would be detrimental. It could be argued that a physiologically meaningful elevation in mHSP72 is one that allows the cell to meet the demands of any additional stress exposure (provided the stressor is of sufficient magnitude to initiate the stress response).

For example, if a stressor induces 100% more mHSP72 than is present in the cell in order to maintain/restore homeostasis and prevent downstream adverse events (for example heat stroke, Selkirk et al., 2008), then a preconditioning response that elicited increases in basal stores near to this critical value (a 100% increase in basal content in this example) would be required in order to fully ameliorate the cellular stress response. However, such a magnitude

of basal increase is unlikely after only one stress exposure. The results obtained in this thesis indicate that acute hypoxic exercise lead to an increase in mHSP72 of approximately 132%, whereas acute heat exercise induced increases of > 160%, with the elevated values persisting for > 24 hours on each occasion. Thus the critical mHSP72 value with regard to acute hypoxic exercise is one that equates to near 132% post exercise increase. Any increase in basal mHSP72 that is near the critical value could be said to be relevant in attenuating the post exercise HSR and improving a cells ability to deal with a stressor. Both acute heat exposure (Chapter 4) and repeated heat (Chapter 5 and 6) and hypoxic exposure (Chapter 6) induced increases in basal mHSP72 near to the hypothesised critical value. Subsequently, mHSP72 was attenuated post hypoxic exercise.

However as 40°C heat stress provided a greater HSP stimulus and magnitude of induction it is conceptually the more efficient stressor in relation to both increasing basal mHSP72 and improving cellular tolerance to 3000m asl. To test this theory, a more severe level of hypoxia (provided this also produced a greater post exercise increase in mHSP72) could be used in the intervention period and then compared to a 40°C heat exposure. Figure 7.4 considers all the data with regard to changes in basal mHSP72 collected throughout this thesis. The forest plot in panal A reveals the the initial PC bout (Chapter 4) had a greater effect on increasing basal mHSP72 than STHA, LTHA or HA immediately prior to the post intervention HST. However these resting samples were taken 24 hours post PC bout compared to 48 hours after the final acclimation period (Chapter 5 and 6) and may not be truly representative of a basal change *per se,* instead reflecting mHSP72 levels still elevated from the PC exercise bout.

It was not possible to conduct an investigation into the time course of the mHSP72 response. It is likely that at 24 hours after the final acclimation day (Chapter 5 and Chapter 6) basal mHSP72 was similarly elevated to values seen in Chapter 4, and began to decrease over the

next 24 hours. Investigating the decay of elevated basal mHSP72 following a PC or acclimation bout would be a useful area of study when determining the optimal time point to apply a preconditioning period or time post acclimation performances.

Although the post exercise mHSP72 response is clearly greater post heat stress (Figure 7.3), the effect size for increases in basal mHSP72 values following STHA, LTHA and HYP ACC show very little difference (Figure 7.4, Panal A). When changes in mHSP72 are examined over the course of acclimation (Figure 7.4, Panal B), both STHA and LTHA are shown to induce a bigger overall effect on basal mHSP72 than HYP ACC, which reaches a plateau following 6 days of LTHA. This data suggests that strategies which induce the greatest change in basal mHSP72 induce the greatest effect on reducing the cellular stress experienced during hypoxia. The effect changes in mHSP72 has on cellular tolerance and physiological function could begin to be investigated by manipulating basal or post exercise HSP72.

Recently two nutritional compounds have been shown to blunt or increase the HSR in humans, and therefore can be used to manipulate basal HSP72 levels prior to a stress exposure. Pope Mosely's group were able to demonstrate that the flavanol, quercitin, blunted the accumulation of HSP72 throughout a period of heat acclimation in humans, apparently affecting broader physiological adaption. For example the classical signs of heat acclimation (reduced exercising T_{core}, HR and PSI) were not present following acclimation under quercitin supplementation. These results therefore suggest a link between cellular tolerance and the acquisition of the acclimated phenotype (Kuennen et al., 2010).

Conversely, glutamine was shown to up-regulate PBMC derived HSP72 prior to an acute period of intense exercise (60-mins running at 70% V̇O2max in 30°C; Zuhl et al., 2014). Consequently, post exercise HSP72 was attenuated and the stress induced permeability of the intestine reduced in parallel with lower levels of plasma endotoxins and pro-inflammatory

cytokines (Zuhl et al., 2014). Studies that utilise these nutritional agents to manipulate the HSR and basal HSP72 would shed further light on the relationship between alterations in molecular markers of adaptation, and whole body physiological function.

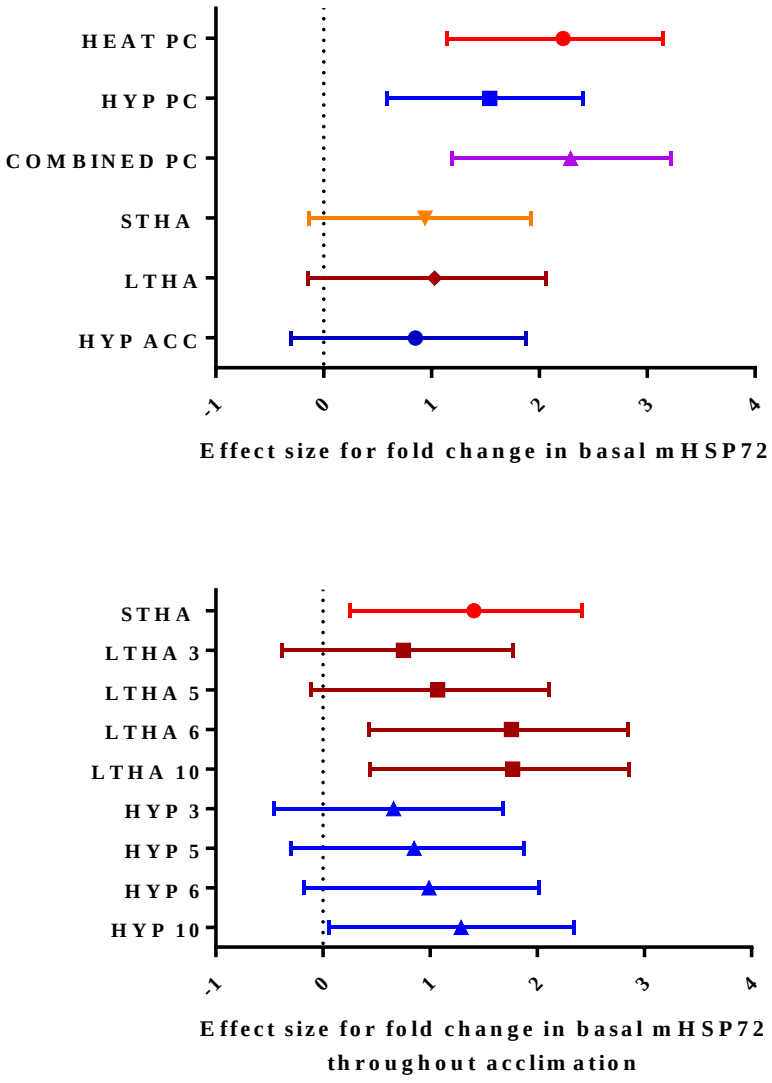

Figure 7.4. Effect size ± 95% confidence intervals for the intervention induced changes in basal mHSP72 prior to a HST (Panal A). Panel B presents effect size ± 95% confidence intervals for the changes in basal mHSP72 throughout an acclimation period.

The results regarding eHSP72 are less consistant. Hypoxia appears to lead to an increase in eHPS72, though to a much smaller magnitude than observed following acute heat stress. The increases in eHSP72 seen post heat exercise have been commonly observed (Marshall et al., 2006; Yamada et al., 2007; Periard et al., 2012; Gibson et al., 2014), however the increases seen post hypoxic exercise have not previously been reported. It has previously been shown that a minimum endogenous criteria is required to induce eHSP72. This criteria – a sufficient change in the absolute (Amorim et al., 2008) and rate of core temperature increase (Gibson et al., 2014) and significant sympathetic activity (Whitham et al., 2006; Whitham et al., 2007) can be achieved via exercise, a thermal environment, or a combination of the two. That participants in the hypoxic conditions achieved heart rates >150beats·min^{-1} and final rectal temperatures of ~38°C suggests they may have met this endogenous criteria. Examining the role hypoxia alone has on eHSP72 release and accumulation would require an experimental design that controlled both abosulte and rate of T_{core} rise, aand sympathic activation. The greater increases in post exercise eHPS72 after heat stressor versus hypoxia are likely a result of the greater magnitude of heart rate and rectal temperature responses, or the earlier surpassing of the minimum endogenous threshold for release into the circulation via exosomal (Multhoff and Hightower, 1996; Lancaster and Febbraio, 2005), ATP (Ogawa et al., 2011) or hormone receptor mediated pathways (Johnson and Fleshner, 2006).

An interesting molecular response that has not been previously noted in humans was the increase in plasma HIF1-α following heat acclimation - a feature that is well established in animal models (Maloyan et al,. 2005; Tetievsky et al,. 2008) of acclimation. This finding further indicates that a shared cellular mechanism of adaption may exist between heat and hypoxia, which primes the exercising human for the rigors of hypoxic exercise. Future studies in which plasma HIF1-α and intracellular and mRNA HIF-α are quantified would

allow this hypothesis to be tested. It was beyond the budget of this present study to investigate downstream markers of HIF-1α activation such as EPO and VEGF in response to heat acclimation.

Figure 7.5 shows forest plots for the effect size data (Cohens *d* ± 95% CI) for mean exercise responses during all post intervention HSTs relative to the pre intervention HST for selected variables measured during each experimental chapter. These data show that the physiological variables typically accociated with heat adaptation (HR, T_{core}, PSI) were consistently reduced following a heat intervention (HEAT, STHA, LTHA) but unaffected by hypoxic stress. The magnitude of this effect appears to increase the longer the adaptive process is applied. LTHA appeared to then have an effect on reducing mean T_{skin} and T_{body} during the hypoxic work bout. It is worth noting that the HYP ACC period had little effect on any measured physiological variable during the post intervention HST, providing more support to the notion that heat-stress at the levels and durations studied offer more physiological benefit during later hypoxic exercise bouts. However the interaction between the molecular responses and physiological responses could not be meaningfully examined during the present series of studies therefore causation between improved cellular tolerance and enhanced physiological responses cannot be inferred.

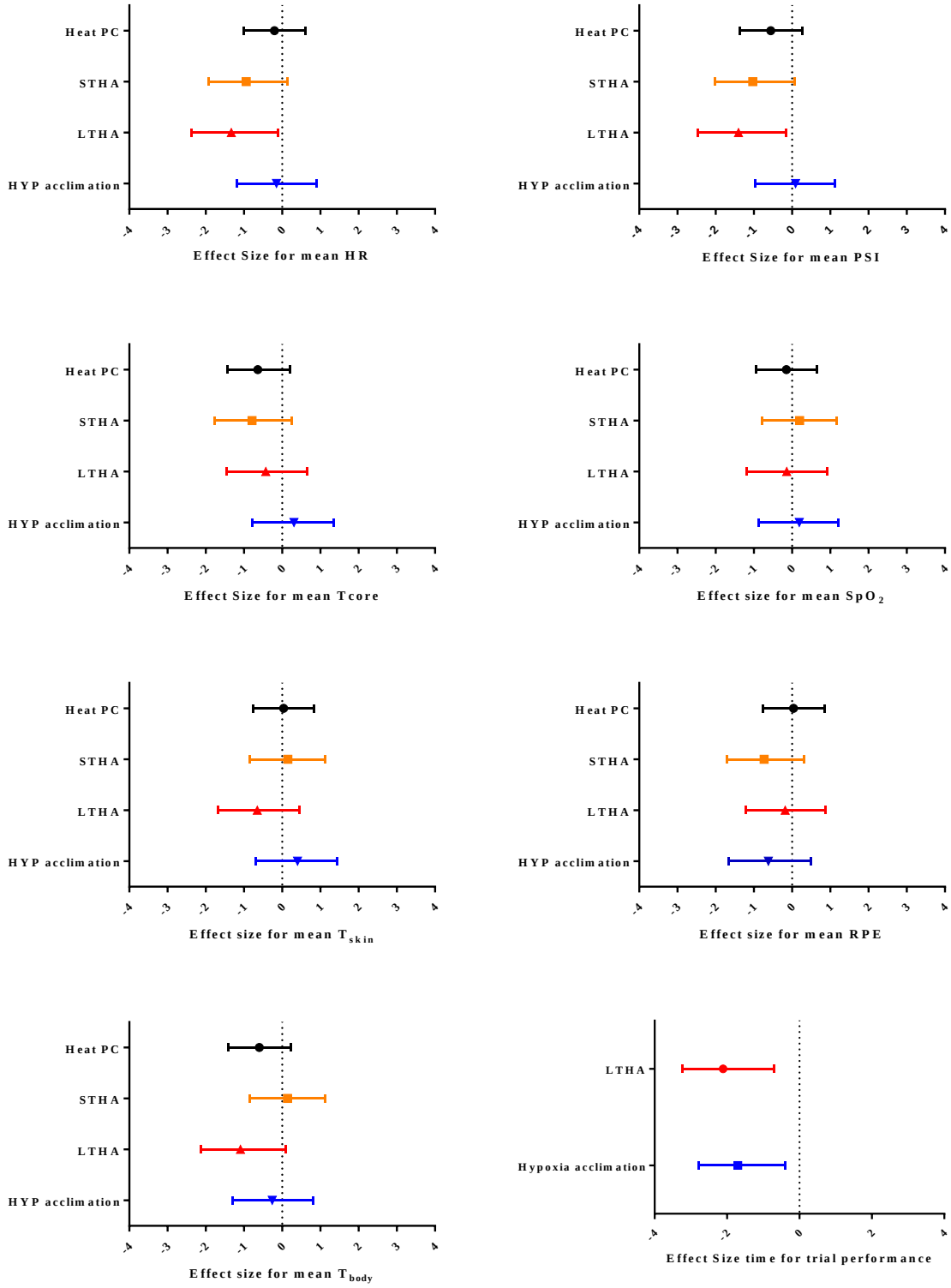

Figure 7.5. Forest plots of effect size data (Cohen's *d* ± 95% confidence intervals) for mean exercise heart rate, T_{core}, T_{skin}, T_{body}, PSI, SpO_2, RPE and time trial time during each experiment.

7.3 LTHA improves aerobic performance in hypoxia

Previous research has demonstrated that a similar mode of heat acclimation employed in this thesis improved the aerobic profile of heat acclimated participants, as assessed by a rightward shift in the blood lactate curve during an incremental cycle test, and improve time trial performance when under both heated and thermoneutral conditions (Lorenzo et al., 2010, 2011). Chapter 6 sought to determine whether a similar period of heat acclimation, as utilized by Lorenzo et al., (2010), would lead to improvements in physiological tolerance when performing both constant load exercise in hypoxia, and completing a performance test (16km TT). The effects this period of acclimation had on 16km TT performance represents the first attempt to assess the impact of heat acclimation on hypoxic exercise performance.

The strength of this study was that it implemented both a normoxic control group and a hypoxic acclimation group. This approach allowed the cellular adaptive response to matched work (60 minutes cycling, 50%N $\dot{V}O_{2\ peak}$) to be compared between these stressors. The study demonstrated that heat acclimation *per se* appeared to offer greater benefits (reductions in exercise HR and attenuated mHSP72 responses) during submaximal hypoxic exercise than an identical period of hypoxic acclimation over the short period of exercise examined (40min, Figure 7.5). Cycling time trial performance was improved in normobaric hypoxia following a 10-day hypoxic training period (d = -1.70; 95% CI = -2.78 - -0.40, and also improved following 10 days of heat acclimation (d = -2.11 95% CI = -3.24 - -0.70; Figure 7.5). The volunteers in the heat group tended to select a higher power output at the start of the TT period compared to their pre-acclimation TT, which persisted for the first half of the time trial. This may indicate that perceptual cues influenced the initial phases of the TT in this group, maybe as a result of the lower heart rate and T_{core} during the initial tolerance phase of the test.

The present series of investigations were only able to utilize normobaric hypoxia due to the available equipment. There have been a recent series of discussions presenting the divergent stances on whether the physiologic responses to normobaric and hypobaric hypoxia are similar or different (Millet et al., 2012; Mournier et al., 2012). Researchers have reported 20-30% increases in resting ventilation while in normobaric hypoxia (Muza et al., 2007), while others reported no differences up to altitudes of 4000m. Recently cycling performance decrement has been shown to be greater in hypobaric hypoxia than in normobaric hypoxia (Beidleman et al., 2014). Thus the effect heat acclimation may have in "real world" mountain environments has yet to be determined and would be an interesting area of future study.

7.4 Experimental limitations and future recommendations

The experimental findings presented in this thesis are novel, however they have been observed in relatively small sample size groups. This dictates that the novel findings require further and extensive investigation, with expanded participant numbers. There is an absence of power calculations within this thesis. The primary reason for this was the absence of data on which to conduct such an analysis. For example, only a limited number of studies had employed the flow cytometry assay utilized within the present series of experiments. In the final experimental chapter (Chapter 6) logistical and economic reasons restricted the number of participants per experimental group to a maximum of 10. Unfortunately, due to a lack of participant interest and participant adherence the study was completed with 3 groups of 7 participants. Due to the widespread criticism regarding retrospective power calculations (Hoenig and Heisy, 2001; Lenth, 2001; Kraermer et al., 2006; Walters et al., 2009), such calculations have not been included. Power calculations should be performed with specific research questions in place (Bacchetti, 2010; Dorey, 2011), therefore the data presented herein regarding mHSP72 could be informative for power calculations for other researchers

wishing to utilize the flow cytometry technique to examine this protein's responses to similar environmental stressors. In small *n* studies the false discovery rate when $P = 0.05$ is unlikely to be 5% and may be at least 30%, or as high as 80% for studies of this nature (Valen-Johnson, 2013; Colquhoun, 2014). It is therefore important that replication studies are carried out to confirm any observations in trials of the sizes conducted in this thesis.

There was considerable variation in the physical characteristics of the participants utilized across each investigation. Whilst habituated trained cyclists were sought, this was not always feasible due to the demanding testing regimes and in particular the considerable demand on participant time during each study. Thus the participants were exclusively drawn from the Coventry University student and staff population. This did however allow for tight experimental controls to be in place regarding the pre-testing diet and standardized time of d0ay for each trial requiring mHSP72 analysis. In Chapters 4 and 5, in which different participant groups were utilized care was taken to ensure adequate matching between groups. Similarly, adequate familiarization to protocols was used throughout experiments in this thesis to negate any potential learning effects. Future studies could revisit these protocols employing a more homogenous group of highly trained participants.

This thesis addressed the experimental aims set (Section 7.1 for summary), providing some evidence for exercise heat stress acting as a preconditioning tool when later exposed to an acute exercise bout in normobaric hypoxia. The results also provide some evidence that mHSP72 can be a marker for improved tolerance. However, cause and effect cannot be established from these experiments. For example, in Chapter 4, the induced increase in mHSP72 and associated reductions in physiological strain are likely not causal. To establish the role mHSP72, and indeed other members of the heat shock family, play in any preconditioning response would require an experimental design in which basal or post

exercise levels can be manipulated. This can be achieved with quercitin (blunting the HSR, Kuennen et al., 2010) or Glutamine (elevated basal HSP72, Zuol et al., 2014) supplementation prior to any intervention aimed at increasing basal mHSP72. If blunting of the mHSP72 response occurs in concert with removal of any physiological preconditioning response, then cause and effect could begin to be established. Likewise, if the upregulation of mHSP72 following glutamine ingestion alters physiological function to a stressor (e.g. acute hypoxia), then the role of the HSR and mHSP72 can be explored more fully.

An investigation into further members of the heat shock protein family would also provide a greater insight into the broader *in-vivo* HSP response to varied environmental stressors. To date no studies have investigated each of the PBMC, muscle and eHSP72 responses together in response to an environmental exercise stress stressor, likely due to the high experimental costs and logistical difficulty of such work. However completion of such projects would allow us to gain a more global perspective of whole body and cellular physiological responses to exercise stress.

7.5 Future experimental objectives

There is evidently a plethora of potential research studies, experimental designs and experimental objectives that are warranted in response to the novel findings presented within this thesis. The aforementioned findings give rise to 4 key questions that require further investigation 1) What role does aerobic fitness play on response to heat/hypoxia and does this moderate the PC response? 2) What are the physiological consequences of increased basal mHSP72 and a blunted HSR to a stressor? 3) Does a threshold mHSP72 response to a stressor exist? 4) How does physiological strain per se impact upon molecular and physiological acclimation to a stressor and what impact does this have on cross-acclimatory responses?

This section will present some example research studies that are viewed as a priority given the data presented in this thesis.

Due to the small sample size and variation in participant characteristics in Chapter 4, further investigation into the whole body physiological responses to combined heat and hypoxic stress and subsequent preconditioning responses is warranted,. Due to logistical and time constraints, it was not possible to recruit a large sample of physically fit ($\dot{V}O_{2\ peak} > 55ml \bullet kg^{-1} \bullet min^{-1}$) and unfit participants ($\dot{V}O_{2\ peak} < 40ml \bullet kg^{-1} \bullet min^{-1}$) to enable a grouped comparision. It is likely that the physiological responses and tolerance to individual and combined stressors would be very different between such groups, and that this may impact upon subsequent preconditioning effects due to the reduced time spent exercising during the initial exposure. Therefore a study in which the initial exposure to a stressor is time-matched (e.g. all exposures 75 minutes) between conditions would be of value. In hindsight, a repetition of this study using such a group would also have allowed a performance test to be incorporated into the HST 24 hours later. Revisiting this study would also allow for the dietary manipulation of HSP72, as previously described, to be factored into the experimental design and begin to address the physiological relevance of the mHSP72 response.

Determining the effects an acute prior exposure to heat, or heat combined with hypoxia, has on exercise performance in acute hypoxia may have important practical applications in both military and athlete populations. This thesis was only able to address the effects heat/heat acclimation conveyed to an acute hypoxic exposure. As sojourns to altitude often occur over a period of days to weeks, often involving slow accent rates, it would be pertinent to determine what effect on performance a prior period of heat acclimation has on longer-term adaption to hypoxia. It could be possible that in the long term the divergent hematological adaptations (plasma volume expansion in the heat versus plasma volume contraction in

hypoxia) between these stressors would actually mean that prior heat acclimation may impair both short and longer-term adaptation to hypoxia and therefore subsequent physical performance.

The acclimation studies used within this thesis incorporated an ecologically valid fixed work protocol. However it is accepted that the isothermic method of heat acclimation, in which daily thermal load is kept constant (e.g. the time above a T_{core} of 38.5 – 39.0°C manipulated) delivers a more complete physiological and cellular adaptation (Taylor et al., 2001; Magalhaes et al., 2010). The same approach could also used within hypoxia via clamping SpO_2 at a fixed level. Individual responses to fixed F_IO_2 has considerable inter-person variability, thus using a fixed level of SpO_2 and therefore an individualized acclimation F_IO_2 would ensure some control over the variation in response is achieved. By maintaining, or even increasing the forcing function (e.g. increasing the target T_{core} or decreasing the SpO_2), the threshold level for a heat shock response is also maintained or increased. In doing so the a thorough examination of the mHSP72 response, and thresholds of induction could begin to be investigated. The results in this thesis indicate that physiological strain has an important role to play in any adaptation process, and therefore would be of import to cross-acclimation. Manipulating the stressor intensities in order to induce similar degrees of overall strain (assessed via heart rate and cellular responses) would allow this question to be addressed. An experiment in which isothermic heat acclimation/clamped SpO_2 are utilized could offer a more mechanistic model in which to assess cross acclimation.

7.6 Practical recommendations

The data presented within this thesis consistently shows that heat is a more efficient stressor when applied as a training aid as increased temperatures elicit greater physiological strain for a lower relative workload compared to hypoxia.

That a heat stress of 40°C, 20% RH reduces $\dot{V}O_2max$ by ~5% versus reductions of ~10-20% at an F_IO_2 of 0.14 provides those using heat training with a greater reserve regarding workrate intensity, thereby allowing for the completion of more intense training sessions. Furthermore, training zones and intensities of training can be better maintained in the heat than hypoxia requiring little or no adjustment to a training period whilst allowing for a greater stress to applied. When individuals are looking for an adjunct to their training then heat may be a cheaper and more effective means of increasing training difficulty and adaptation than a period of normobaric hypoxia.

The area of heat-hypoxia cross tolerance in humans has yet to be fully explored. Practical recommendations regarding the use of acute or repeated exercise-heat bouts to prepare for altitude sojourns should be avoided until the exercise-heat stress PC /acclimation responses have been examined in hyperbaric-hypoxic conditions. The considerable individual variation in response to both heat and hypoxic adaptation would also need to be more thoroughly considered within participant cohorts prior to the dissemination of practical advice.

7.7 Conclusion

These studies show that both acute and repeated exposures to an exercise heat stress of 40°C, in which T_{core} is elevated to modest peaks of < 39°C induces a greater mHSP72 response than acute or repeated hypoxia equivalent to 3000m asl. Whether the increased mHSP72 or increased physiological strain experienced in the heat compared to hypoxia, is the driver for preconditioning and cross acclimation could not be established within this thesis. However, these results indicate the potential for exercise heat stress in alleviating physiological strain and improving exercise performance in acute moderate hypoxia. Further studies investigating the mechanisms behind this cross acclimation, in both the laboratory and field settings are required in order to produce specific guidelines prior to undergoing altitude

sojourns. That heat is more readily accessible than specialist hypoxic equipment indicates that that training in elevated temperatures could be a cheap and time efficient method in alleviating the negative performance implications associated with acute exposure to altitude.

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